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CHRONIC HORMONAL CONTROL OF LIPID SYNTHESIS AND HYDROLYSIS IN ADIPOCYTES

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A thesis presented for the degree of Ph.D. at the University of Glasgow

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Abstract

The aim of this study was to further elucidate the mechanisms whereby growth hormone (GH) exerts its chronic effect on adipose tissue metabolism, and in particular the effects of GH on lipolysis. Previous studies had shown that tumour necrosis factor alpha (TNF α) chronically increases basal lipolysis in rat epididymal adipocytes: an effect that is similar to that of GH. Other research had implicated a protein with a half-life of less than 3 hours in the regulation of lipolysis and lipogenesis by GH. This led to the investigation if TNF α might be this putative protein involved in mediating the chronic metabolic effects of GH. Initial studies used ovine adipose tissue explants. TNF α caused a small increase in basal lipolysis and attenuated insulin effects on lipogenesis. However, the effects of TNF α were smaller than those of GH and TNF α did not appear to mimic the effects of GH on isoproterenol-stimulated lipolysis in this system. Therefore TNF α was not the protein involved in GH regulation of lipolysis and lipogenesis.

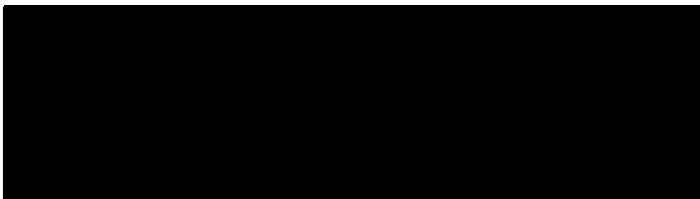
Previous studies in the laboratory on the mechanism of GH action had used inhibition of signal transduction components in an explant system. More specific effects could be observed by using an antisense approach, but this required the use of a cell culture system rather than adipose tissue explants. The suitability of an ovine cell culture system was established for investigating the molecular basis of the lipolytic effects of GH; in particular the inhibitory effects of GH on adenosine inhibition of lipolysis. The lipolytic system partially developed in primary ovine adipocytes, but the antilipolytic system did not appear to develop. However, by manipulating the differentiation conditions, I significantly improved both cell differentiation and the lipolytic response

and sensitivity to isoproterenol, but there was no improvement in response to adenosine. As an alternative, the suitability of the murine cell line, 3T3-F442A, was investigated for determining the molecular basis of the lipolytic effects of GH. However, although the lipolytic system did develop in differentiated 3T3-F442A adipocytes and response to isoproterenol was observed, the antilipolytic system did not appear to develop either. This line of investigation was not pursued further.

Therefore, I decided to investigate the effects of GH and insulin on the lipogenic system in 3T3-F442A adipocytes instead, with a view to extending previous observations by others (Millar, 1998) in the laboratory on the roles of specific isoforms of protein kinase C (PKC) on the modulation of lipogenesis by insulin and GH. The main objective was to determine the role of PKC isoforms in the modulation of the effect of insulin and GH on activation and expression (mRNA) of the lipogenic enzyme acetyl CoA carboxylase (ACC). However, the effect of the hormones on lipogenesis, and especially ACC, was considered to be too small to investigate the roles of specific PKC isoforms, despite trying many different ways of improving the hormone effects. A possible explanation for the poor response to insulin was that the lipogenic system was not "switching off" in the absence of insulin, so isoproterenol was added to the 3T3-F442A adipocytes to decrease lipogenesis. Isoproterenol did reduce the rate of lipogenesis, but the effect of insulin was still small. Therefore, modulation of the effect of specific phosphodiesterase (PDE) isoforms on lipogenesis was explored as an alternative. The use of specific PDE inhibitors showed that both PDE3 and PDE4 enzymes were involved in the modulation of lipogenesis in 3T3-F442A adipocytes.

Declaration

This thesis has been compiled by myself and is based on work carried out on an original line of research. This thesis has not been offered in any previous application for a degree. All sources of information are referenced and help provided by other people has been duly acknowledged.



Shona E. Melrose

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List of Abbreviations

AA	arachidonic acid
AC	adenylate cyclase
ACC	acetyl coA carboxylase
Acrp30/AdipQ	adipocyte complement-related protein
ADP	adenosine diphosphate
ANOVA	analysis of variance
AP-1	activating protein-1
APRF	acute-phase response factor
ATP	adenosine triphosphate
bGH	bovine growth hormone
BSA	bovine serum albumin
C/EBP	CCAAT/enhancer binding protein
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanine monophosphate
Cil	cilostamide
DAG	diacylglycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
FAS	fatty acid synthetase
FCS	foetal calf serum

G-3-PDH	glycerol-3-phosphate dehydrogenase
GH	growth hormone
GHBP	growth hormone binding protein
GH-R	growth hormone receptor
GLUT	glucose transporter
GPDH	glycerol 3-phosphate dehydrogenase
Grb2	growth factor receptor-bound protein 2
GTP	guanine triphosphate
H7	[1-{5-isoquinolinesulfonyl}-2-methyl piperazine, HCl]
HBSS	Hank's balanced salt solution
Hepes	(N-[2-hydroxyethyl]piperazine-N'-[4-butanedisulphonic acid])
hGH	human growth hormone
HSL	hormone-sensitive lipase
IBMX	3-isobutyl-1-methylxanthine
IGF-1	insulin-like growth factor-1
IL	interleukin
INS	insulin
IR	insulin receptor
IRS	insulin receptor substrate
ISGF3	interferon-stimulated gene factor 3
JAK	Janus tyrosine kinase
JNK	N-terminal kinase
LPL	lipoprotein lipase
MAP kinase	mitogen-activated protein kinase
MBP	myelin basic protein

MCV	mean cell volume
MEK	mitogen-activated protein kinase kinase
MGF	mammary gland factor
MIF	macrophage migration inhibitory factor
mm-IBMX	8-methoxymethyl-IBMX
mRNA	messenger ribonucleic acid
MTT	[3-{4,5dimethylthiosal-2-yl}-2,5-diphenyl-tetrazoliumbromide]
NAD	nicotinamide adenine dinucleotide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide hydrogen phosphate
NCS	newborn calf serum
NF	nuclear factor
oGH	ovine growth hormone
PAI-1	plasminogen activator inhibitor factor-1
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDE	phosphodiesterase
PDH	pyruvate dehydrogenase
PGE	prostaglandin E
PH	pleckstrin homology
PI	phosphatidylinositol
PI-3kinase	phosphatidylinositol 3-kinase
PIA	N ⁶ -phenylisopropyladenosine
PKA	protein kinase A
PKB	protein kinase B

PKC	protein kinase C
PKD	protein kinase D
PLA ₂	phospholipase A ₂
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PPAR	peroxisome proliferator-activated receptor
REML	residual maximum of likelihood
Rol	rolipram
SAPK	stress-activated protein kinase
SEM	standard error of the mean
SH2	Src homology-2
SHPTP	SH2-containing phosphate tyrosine phosphatase
SIE	sis-inducible element
SMase	sphingomyelinase
SOS	son-of-sevenless
STAT	signal transducers and activators of transcription
T ₃	triiodothyronine
TNF-R	tumour necrosis factor receptor
TNF α	tumour necrosis factor alpha
TZD	thiazolidinedione
VLDL	very low density lipoprotein

CHAPTER ONE

INTRODUCTION

1.1 General

White adipose tissue occurs in a number of distinct depots throughout the body, at least some of which have specialised roles. Adipose tissue is important in internal metabolism, especially in relation to storage and mobilisation of fatty acids. Both the amount and the topographical localisation of the adipose tissue seem to be of importance for the health consequences of obesity. Reserves of lipid in adipose tissue have a vital role in mammalian physiology, and are particularly important for successful lactation in most species, including domestic ruminants in which milk yield increases initially more rapidly than food intake (Bauman & Currie, 1980). This results in a transient period of negative energy balance, when use of reserves of stored nutrients becomes very important (Vernon & Flint, 1984). Lipolysis (hydrolysis of triglycerides to fatty acids) is increased during lactation due to changes in the concentrations of acutely acting factors, and in the ability of adipocytes to respond to them. Decreased lipid synthesis (esterification of fatty acids to triglycerides) in addition to enhanced lipolysis leads to substantial loss of adipose tissue lipid reserves during lactation (Chilliard 1987; Vernon, 1989; McNamara, 1991). The factors responsible have not been fully resolved, but insulin and growth hormone have been implicated in ruminants, and insulin and prolactin in rats and other laboratory species (Chilliard 1987; Vernon, 1989; McNamara, 1991).

Obesity is strongly linked with insulin resistance, and is a major cause of morbidity in the developed world (Reaven, 1988; DeFronzo *et al.*, 1992). Therefore, factors regulating adipose tissue metabolism have been investigated with increasing enthusiasm over the past two decades.

1.2 Adipose tissue

In lower vertebrates such as fish and most amphibians and reptiles, adipose tissue occurs in a few large depots in the abdomen. There, it can undergo extensive changes in mass without affecting the position of the animal's centre of gravity (Pond, 1978). Fish, amphibians and reptiles are all poikilotherms, and so have relatively low energy requirements, thus they need only limited amounts of adipose tissue. The evolution of homeotherms greatly increased the energy requirements of animals, so in mammals we find large amounts of white adipose tissue. Thus, a highly developed adipose tissue system is characteristic of all mammalian species (Pond, 1984). The major form of adipose tissue in adult mammals is white adipose tissue, which is found in distinct sites throughout the body and is located mostly in the abdominal cavity, under the skin (subcutaneous depots) or within the musculature (inter- and intra-muscular depots) (Pond, 1992). In some animals, adipose tissue can constitute as much as 50% of the body weight, and in some grotesquely obese humans it can be even higher (Flint & Vernon, 1993). Obesity is characterised by an increased fat mass and occurs when the intake of food exceeds the energy requirement of the body for a sustained period (Spiegelman, 1996). In all mammals, there is very little variation in the pattern of distribution of fat depots, and appears to have occurred early in mammalian evolution (Pond & Mattacks, 1985).

Mature adipocytes are the main cellular component of white adipose tissue, and are uniquely equipped to function in energy storage and balance under tight hormonal control. Mature adipocytes make up approximately 25% of the cell population, and are formed from precursor cells which proliferate and differentiate into mature adipocytes (Ailhaud *et al.*, 1992; Flint & Vernon, 1993; Grégoire *et al.*, 1998), but

adipocytes themselves do not divide. The stromal-vascular fraction of adipose tissue contains endothelial cells, mast cells and blood cells, as well as these precursor cells at different stages of development (Flint & Vernon, 1993), which can be induced to proliferate and differentiate *in vitro* in chemically defined media.

The main, but not only, function of white adipose tissue is uptake, storage and controlled release of lipids, which are all processes that can involve large and rapid changes in tissue mass. An additional role that adipose tissue plays is that of insulation. For example, in both reindeer and polar bears, it has been shown that after intensive feeding and rapid fattening during the brief arctic summer and autumn, the subcutaneous depots enlarge disproportionately, and may become more than 100mm thick over the rump and hind-quarters (Pond *et al.*, 1993). Their findings suggest that all the skin-side of the adipose tissue is normally cooler than the inner side, as expected on the insulation hypothesis, and that the composition of the storage lipids is adapted to this situation. However, the theory that subcutaneous adipose tissue is for insulation is not always the case, as in most land animals and ducks this fat is the first to be utilised in conditions of poor nutrition (see Flint & Vernon, 1993). Adipose tissue that surrounds vital organs may also serve as protection. It has also been suggested that cardiac and some intermuscular adipose tissue depots may play a critical, localised role in providing fatty acids as a source of fuel for adjacent muscle fibres (Marchington *et al.*, 1989). Adipose tissue plays a role in buoyancy in many species of fish.

Several lines of evidence indicate that adipocytes can function as endocrine cells, releasing not only fatty acids, but also a variety of bioactive peptides (Flier, 1995) and

other factors (Richelson, 1992; Mohamed-Ali *et al.*, 1998). Production of such secreted signalling molecules varies between adipocytes in different adipose tissue depots. For example, leptin, the adipocyte-specific product of *ob* gene that plays a major role in the control of body weight in rodents (Zhang *et al.*, 1994), is expressed more in the adipocytes from the subcutaneous depots than abdominal depots (Hube *et al.*, 1996; Montague *et al.*, 1997). Adipose tissue is also important for the immune system. Immune system-related proteins produced by adipocytes include adipsin, adipocyte complement-related protein (Acrp30/AdipoQ), tumour necrosis factor alpha (TNF α) and macrophage migration inhibitory factor (MIF); leptin is also required for efficient functioning of the immune system (Lord *et al.*, 1998). Vasoactive peptides that are secreted by adipocytes include angiotensinogen and plasminogen activator inhibitor type 1 (PAI-1), and white adipose tissue contains all the main components of the renin-angiotensin system (Jonsson *et al.*, 1994). Angiotensinogen could play a role in regulating adipose tissue blood supply and fatty acid efflux from fat (Frederich *et al.*, 1992), while PAI-1 may be involved in the development of vascular diseases associated with abdominal obesity (Alessi *et al.*, 1997; Lundgren *et al.*, 1996; Shimomura *et al.*, 1996).

The other type of adipose tissue is brown adipose tissue. This is more prevalent in young animals, and has an important role in heat production by non-shivering thermogenesis. It is typically found in a small number of defined depots (Himms-Hagen, *et al.*, 1972) and has dense blood and nerve supplies (Fawcett, 1952; Ballard, 1974), as well as large numbers of mitochondria and the protein thermogenin (Cannon *et al.*, 1982). These differences, as well as others found between white and brown adipose tissue, indicate the major functional distinction between these tissue.

However, this project was concerned with white adipose tissue, so all following information will focus on white adipose tissue only.

1.2.1 Adipose tissue development

In most species, white adipose tissue forms before birth. This has been assessed by morphological studies performed on human, pig, mouse and rat embryos (Desnoyers & Vodovar, 1977; Poissonnet *et al.*, 1983; Poissonnet *et al.*, 1988; Slavin, 1979).

White adipose tissue expansion takes place rapidly after birth as a result of increased fat cell size, as well as an increase in adipocyte cell number. The potential to generate new fat cells persists even at the adult stage.

Several studies on multipotent clonal cell lines have suggested that adipocyte lineage derives from an embryonic stem cell precursor with the capacity to differentiate into the mesodermal cell types of adipocytes, chondrocytes, osteoblasts and myocytes. It has been shown that treatment of a murine embryonic cell line C3H10T1/2 with a demethylating agent induces areas of muscle, cartilage and fat cells (Konieczny & Emerson, 1984; Taylor & Jones, 1979), and so the C3H10T1/2 cell line may represent multipotential stem cells that are blocked at the mesodermal pathway.

For the past two decades, *in vitro* systems have been extensively used to study adipocyte differentiation. Various pre-adipose cell lines and primary cultures of adipose-derived stromal vascular cells have been used from rats (Björntorp *et al.*, 1980; Deslex *et al.*, 1987; Wiederer & Löffler, 1987; Sztalryd *et al.*, 1989, 1991; Grégoire *et al.*, 1990; Kirkland *et al.*, 1990, 1996; Sztalryd & Faust, 1990; Wabitsch *et al.*, 1996), pigs (Hausman *et al.*, 1992), humans (Hauner *et al.*, 1988; Hauner &

Entenmann, 1991) and sheep (Soret *et al.*, 1999). Pre-adipose cell lines, as well as primary pre-adipocytes, are already committed solely to the adipocyte lineage, although they may represent different stages of adipocyte development. The most frequently used cell lines are 3T3-L1 and 3T3-F442A. These were clonally isolated from Swiss 3T3 cells derived from disaggregated 17 to 19 day mouse embryos (Green & Meuth, 1974; Green & Kehinde, 1975; Green & Kehinde, 1976). Ob1771 cells from dedifferentiated adipocytes have also been utilised to study adipocyte differentiation (Doglio *et al.*, 1986). However, most of the mechanisms that have been elucidated have been found using these cell lines.

The committed pre-adipocyte maintains the capacity for growth, but has to withdraw from the cell cycle (growth arrest) before adipocyte conversion. Acquisition of the adipocyte phenotype is characterised by chronological changes in the expression of numerous genes during adipocyte differentiation, which take place mainly at the transcription level, although post-transcriptional regulation occurs for some adipocyte genes (Wilkison *et al.*, 1990; Moustaid & Sul, 1991). As well as the activation of genes, those genes that are inhibitory to adipogenesis are repressed. Two transcription factors, CCAAT/enhancer binding protein alpha (C/EBP- α) and peroxisome proliferation-activated receptor gamma (PPAR- γ) have been shown to transactivate adipocyte specific genes. PPAR- γ is largely adipocyte-specific, and is expressed at low but detectable levels in pre-adipocytes. Its expression rapidly increases after hormonal induction of differentiation, and maximum levels of expression are attained in mature adipocytes (Brun *et al.*, 1996; Chawla & Lazar, 1994). Both C/EBP- α and PPAR- γ also appear to be involved in the growth arrest that is required for adipocyte

differentiation (Umek *et al.*, 1991; Timchenko *et al.*, 1996; Altioek *et al.*, 1997). After growth arrest at confluence, pre-adipocytes must receive an appropriate combination of mitogenic and adipogenic signals to continue through subsequent differentiation steps. Studies on pre-adipose cell lines have shown that growth-arrested cells undergo at least one round of DNA replication and cell doubling. This has been proposed to lead to the clonal amplification of committed cells (Pairault & Green, 1979). During adipocyte differentiation, cells convert from a fibroblastic to a spherical shape, and dramatic changes occur in cell morphology, cytoskeletal components, and the level and type of extracellular matrix (ECM) components. Decrease in actin and tubulin expression is an early event in adipocyte differentiation that precedes overt changes in morphology and the expression of adipocyte-specific genes (Spiegelman & Farmer, 1982). These changes in cell shape reflect a distinct process in differentiation, and are not the result of accumulated lipid stores. It is likely that these changes could influence the expression and action of PPARs and C/EBPs during adipocyte differentiation. A switch in collagen gene expression is also an early event of adipocyte differentiation (Aratani & Kitagawa, 1988; Weiner, *et al.*, 1989; Darimont *et al.*, 1994). During the terminal phase of differentiation, adipocytes in culture greatly increase *de novo* lipogenesis and acquire sensitivity to insulin. The activity, protein and mRNA levels for enzymes involved in triacylglycerol metabolism increase up to 100-fold (Spiegelman *et al.*, 1983; Paulauskis & Sul, 1988; Weiner *et al.*, 1991). Adrenergic and insulin receptor numbers increase (Lai *et al.*, 1982; Fève *et al.*, 1990; Guest *et al.*, 1990; Fève *et al.*, 1991), as well as the number of glucose transporters (Garcia de Herreros & Birnbaum, 1989), and the adipocytes also synthesise other adipose tissue-specific products (Spiegelman *et al.*, 1983; Bernlohr *et al.*, 1984;

Greenberg *et al.*, 1993; Ibrahimi *et al.*, 1996; Sfeir *et al.*, 1997) and produce a number of secreted products.

It is generally accepted that mature, lipid-filled adipocytes that have progressed beyond a specific stage in adipocyte differentiation are committed to subsequent terminal differentiation, and can neither dedifferentiate nor enter mitosis (Wier & Scott, 1986; Wang & Scott, 1993). However, the precise stage beyond which adipocytes can be considered terminally differentiated is not clearly defined, and the progressive dedifferentiation of mature adipocytes followed by cell division has also been reported (Sugihara *et al.*, 1986).

1.2.2 Adipose tissue metabolism

Food energy in animals is stored primarily as triacylglycerols in lipid storage droplets of adipocytes. The important features of adipocyte metabolism are the synthesis, storage, and subsequent hydrolysis of these triacylglycerols, and the relative rates of their synthesis and breakdown determine the amount of lipid within the adipocyte.

The fatty acids required for triglyceride esterification within the adipocytes are provided by lipogenesis (*de novo* synthesis) or by the triacylglycerols of very low density lipoproteins (VLDL) and chylomicrons. Lipoprotein lipase (LPL) is secreted by adipocytes and is transported to the endothelial cells lining the capillaries and attaches to the outer surface of the plasma membrane facing the lumen of the capillary. LPL hydrolyses the triglycerides of plasma chylomicrons and VLDL. It is thought that transport into the cell of resulting fatty acids and monoglycerols is facilitated by a fatty acid translocase located in the plasma membrane (Scow &

Blanchette-Mackie, 1985). LPL activity (Haugebak *et al.*, 1974; Lee & Kauffman, 1974; Savard & Greenwood, 1988; Rebuffe-Scrive *et al.*, 1989; Björntorp *et al.*, 1990) and the rate of lipogenesis (Lee & Kauffman, 1974; Whitehurst *et al.*, 1981; Fried *et al.*, 1982) vary between adipose tissue depots in ruminants, rats, pigs and humans.

The total rate of lipid accumulation is also dependent on the rate of lipolysis. The mobilisation of fatty acids is catalysed by the enzyme hormone sensitive lipase (HSL), and is under complex acute and chronic endocrine control (Vernon, 1992). Lipolysis, and its response to catecholamines, is also reported to vary between adipose tissue depots in rats (Hartman & Christ, 1978), dogs (Berlan *et al.*, 1982) and humans (Rebuffe-Scrive *et al.*, 1989).

1.2.3 Lipogenesis

The rate of lipogenesis (fatty acid synthesis) is regulated by a number of enzymes, the most important of which is acetyl CoA carboxylase (ACC) which is subject to both chronic (gene expression) and acute control. ACC exists in both active and inactive states, with insulin and catecholamines causing an increase and decrease in the activation status of the enzyme, respectively. Insulin also acts chronically to increase gene transcription and, therefore, the amount of ACC (Vernon, 1992). ACC catalyses the conversion of acetyl CoA to malonyl CoA (Vernon, 1992), and fatty acid synthetase (FAS) converts malonyl CoA to fatty acids. Acetate, glucose and possibly lactate are the most important precursors of acetyl CoA, but it can also be produced from a number of amino acids. There are certain basic differences between ruminants and non-ruminants with regard to fatty acid synthesis. The major precursor for lipogenesis in adipose tissue is glucose in non-ruminants (Ballard *et al.*, 1969),

whereas acetate rather than glucose has been shown to be the principle precursor in adipose tissue from ruminants such as sheep, cattle and goats (Ingle *et al.*, 1972; Whitehurst *et al.*, 1978; Liepa *et al.*, 1978).

NADPH is also required for lipogenesis, and can be derived from three systems; glucose-6-phosphate and 6-phosphogluconate dehydrogenases, malic enzyme and NADP-isocitrate dehydrogenase. Isocitrate dehydrogenase has special importance in ruminants (Vernon, 1980), whilst the importance of the others varies with species.

1.2.4 Lipolysis

White adipose tissue metabolism also involves the hydrolysis of triacylglycerols to glycerol and free fatty acids in the process termed lipolysis, and is under complex acute and chronic endocrine control (Vernon, 1992). Some of the fatty acids released from ester linkage are re-esterified, and are retained in the cell. The rate of lipolysis can be measured by glycerol release, as adipocytes are deficient in the glycerol kinase, and so glycerol produced by lipolysis cannot be phosphorylated and used for re-esterification (Margolis & Vaughan, 1962).

Adipocytes possess both α_2 - and β -adrenergic receptors (Vernon & Sasaki, 1991). Lipolysis is stimulated acutely by catecholamines acting *via* the β -adrenergic receptor and glucagon acting *via* the glucagon receptor, and inhibited by adenosine acting *via* the adenosine A₁ receptor, and also catecholamines acting *via* the α_2 -adrenergic receptor. Responsiveness to the acutely acting factors is modulated by steroid and thyroid hormones, and by GH (Vernon 1992).

Binding of catecholamines to the β -adrenergic receptor in the plasma membrane causes activation and dissociation of the heterotrimeric GTP-binding protein, G_s . This in turn causes activation of adenylate cyclase (AC) which catalyses the synthesis of cAMP from ATP; cAMP activates protein kinase A (PKA). cAMP phosphodiesterases modulate the concentration of cAMP by degrading it to AMP (Bolger, 1994; Beavo, 1995). Insulin activates cAMP phosphodiesterases and so decreases cAMP levels (Conti *et al.*, 1991; Manganiello, 1995). The rate-limiting enzyme, hormone-sensitive lipase (HSL), can be phosphorylated and activated by the cAMP-dependent PKA, so changes in both adenylate cyclase (AC) and cAMP phosphodiesterase activities modulate the rate of lipolysis (Belfrage, 1985). It has been observed that, on lipolytic stimulation, HSL translocates from the cytosol to its substrate on the surface of the lipid storage droplet (Egan *et al.*, 1992). Perilipins are a family of proteins found tightly bound to the limiting surface of the lipid droplet, and are acutely phosphorylated by PKA upon lipolytic stimulation. It has been suggested that both HSL and perilipin redistribute during lipolysis, and that perilipin may serve a role by acting as a barrier to deny access of HSL to its lipid substrate in unstimulated adipocytes (Clifford *et al.*, 1997). HSL hydrolyses triacylglycerides by cleaving two molecules of fatty acids. The resulting monoglycerol is hydrolysed to glycerol and fatty acid by monoacylglycerol lipase (Vernon, 1992). The rate-limiting step is thought to be the cleavage of the first fatty acid moiety by HSL, while monoacylglycerol is normally present in trace amounts in the tissue as monoacylglycerol lipase is very active (Vernon, 1992).

AC activity can be regulated by both stimulatory and inhibitory receptors, which exert their actions through the heterotrimeric G-proteins, G_s and G_i , respectively. G-

proteins comprises an α -subunit, together with β - and γ -subunits. Activation of a heterotrimeric G-protein by an agonist-liganded receptor results in exchange of bound GDP for GTP at the binding site present in the α -subunit, and dissociation of the $\beta\gamma$ complex. The active α -subunit is then able to interact with the catalytic subunit of AC (Levitzki, 1990), and either stimulate or inhibit its activity, which in turn causes an increase or decrease in the rate of lipolysis. The dissociated $\beta\gamma$ complex from G_i possibly also binds to $G_{s\alpha}$ subunits, therefore also inducing the inhibition of AC (Levitzki, 1990; Birnbaumer, 1992; Tang & Gilman, 1992). Adenosine (Vernon *et al.*, 1991) and prostaglandin E (PGE) (Richelsen, 1992) are produced in adipose tissue, and act *via* their own receptors to inhibit lipolysis. Catecholamines can also inhibit lipolysis by this mechanism by acting *via* the α_2 -receptor. Therefore, the activity of HSL at any time is thus determined by the balance struck between stimulatory and inhibitory agonists (Rodbell, 1980).

1.2.5 Hormonal regulation of adipose tissue metabolism

Adipocyte metabolism is regulated both acutely and chronically by a number of hormones and other factors. Lipolysis in adipose tissue is under complex acute control (Vernon, 1992) by both stimulatory (catecholamines, glucagon) and inhibitory (insulin, adenosine, prostaglandins, fatty acids) factors. Growth hormone (GH), glucocorticoids, sex steroids and thyroid hormones chronically control the ability of these acute factors to influence the rate of lipolysis.

GH acts chronically to increase lipolysis and decrease lipogenesis by altering the ability of adipose tissue to respond to acute endocrine and other signals, but it also

seems to have some conflicting actions. GH can exert an acute, transient, insulin-like effect in laboratory species (Goodman *et al.*, 1987; Vernon & Flint, 1989), although the physiological significance of this is unclear as the conditions required (absence of the hormone for a period of several hours) are unlikely to occur *in vivo*. It has also been suggested that GH can have an acute lipolytic response in some species, although this has not been completely resolved; GH does not appear to have an acute lipolytic effect in ruminants and pigs (Boyd & Bauman, 1989; Vernon & Flint, 1989; Etherton & Smith, 1991).

Although the effects of GH on many tissues are mediated *via* insulin-like growth factor-I (IGF-I), this is not the case for adipocytes, where GH exerts its effects directly *via* its cell surface receptor (Vernon & Flint, 1989). GH decreases the rate of lipogenesis in adipocytes *in vivo* by suppressing the transcription of acetyl CoA carboxylase (ACC) (Bauman & Vernon, 1993) and fatty acid synthase (FAS) (Harris *et al.*, 1993) mRNA. The activities of ACC and FAS are also decreased after chronic GH treatment of adipose tissue or adipocytes *in vitro* (Dietz & Schwarz, 1991; Bauman & Vernon, 1993; Vernon *et al.*, 1993). GH also inhibits the activation of ACC by insulin by possibly inhibiting the synthesis of a protein required for the mediation of the insulin-induced activation of the enzyme (Vernon *et al.*, 1991). Chronic treatment with GH suppresses the ability of fat cells to respond to insulin in the absence of any change in their capacity to bind the hormone (Foster *et al.*, 1988). The insulin-antagonistic effects of GH may be important in the development of insulin resistance, which is associated with obesity and diabetes mellitus (Fowelin *et al.*, 1991).

Glucocorticoids have been shown to increase β -adrenergic receptor number, but decrease maximum AC activity in rats (Giudicelli *et al.*, 1989; Ros *et al.*, 1989). Dexamethasone, a glucocorticoid analogue, has also been shown to increase the response and sensitivity to the β -agonist, isoproterenol, and decrease maximum AC activity in sheep adipose tissue (Finley *et al.*, 1990). Prolonged exposure to dexamethasone also increases the response to α_2 -adrenergic agents and to adenosine in sheep adipose tissue (Vernon *et al.*, 1991b). Hypothyroidism in rats has been shown to decrease the response to β -agonists, and increase the response to adenosine and prostaglandin E₂ (Malbon *et al.*, 1988; Vernon *et al.*, 1991a). The effects of adenosine and prostaglandin E₂ were found to involve an increase in G_i activity, and were not due to receptor level changes (Malbon *et al.*, 1988; Milligan & Saggerson, 1990). Hyperthyroidism in rats increased the response to β -agonists and decreased the response to adenosine, due to a decrease in adenosine receptor number (Malbon *et al.*, 1988).

Insulin is the major anabolic hormone of the body (Vernon & Flint, 1989), promoting lipid synthesis and inhibiting lipolysis in adipose tissue, as well as increasing glucose transport and the uptake of other metabolites. Insulin decreases the rate of lipolysis partly by activating a cyclic AMP phosphodiesterase. There is at least one more mechanism involved in the decrease of lipolysis by insulin, and this probably involves the activation of protein phosphatase 2A, resulting in the dephosphorylation of HSL (Yeaman, 1990). It is well established that incubation of adipose tissue with insulin markedly increases the rate of fatty acid synthesis. This involves a series of changes depending on the serum, including enhanced glucose transport and activation of

enzymes such as pyruvate dehydrogenase and acetyl CoA carboxylase. Chronically, insulin causes an increase in the gene expression of lipogenic enzymes. In ruminant animals, in which acetate is the major precursor of fatty acids, activation of ACC is of particular importance in the overall control of lipogenic processes. Increased total ACC activity and the proportion of the enzyme in the active state is also observed after chronic incubation with insulin (Vernon *et al.*, 1991). The signalling pathways of insulin are described in section 1.5.

1.3 Growth hormone

Growth hormone (GH) is a member of a protein family which also includes prolactin and placental lactogen. These genes are believed to have evolved from a common ancestral gene (Miller & Eberhardt, 1983). GH, also known as somatotropin, is synthesised and secreted by the anterior pituitary gland. The secretion of GH from the pituitary gland is regulated by two peptides; growth hormone-releasing factor, which stimulates release, and somatostatin, which inhibits its release (Page *et al.*, 1989)

GH isolated from various vertebrate species has been shown to possess highly conserved structural features (Miller & Eberhardt, 1983; Nicoll *et al.*, 1986; Watahiki *et al.*, 1989). It is a single chain peptide hormone of 191 amino acids (Wallis, 1989), and is "species specific" (for review, see Bauman & Vernon, 1993). Human GH (hGH) and bovine GH (bGH) have only 65% homology, and so bGH cannot bind effectively to the human GH receptor (Wallis, 1989). Ovine GH (oGH) and bGH only differ by one amino acid however, and so bGH is biologically active in sheep. bGH has four naturally produced variants. Recombinant bGH's have extra amino acids at the N-terminus, but are similar to bGH biologically (Bauman & Vernon, 1993).

1.3.1 Growth hormone and lipid metabolism

Downs (1930) and Bierring and Nielson (1932) were first to show that an alkaline extract of the anterior pituitary gland reduced carcass fat in rats. This was verified by Lee and Schaffer (1934), who reported that pair-fed rats injected with a crude alkaline extract of bovine pituitaries not only gained more weight, but also contained proportionally more muscle and less fat. This introduced the idea that GH and the metabolism of lipids were related. In 1945, GH was isolated and purified from the anterior pituitary (Li *et al.*, 1945), which led to the first experiments to be conducted to show that crude preparations of GH could reduce carcass fat in rats (Li *et al.*, 1948). Later studies also gave backing to the fact that prolonged treatment of rats with preparations rich in GH decreased the amount of body fat (Goodman & Schwarz, 1974; Rao & Ramachandran, 1977), while the availability of recombinant GH has shown similar effects in ruminants (Hart & Johnsson, 1986; Boyd & Bauman, 1989; Bauman, 1992; Steele & Evoke-Clover, 1993).

GH is thought to be a homeorhetic hormone regulating the partitioning of absorbed nutrients between different tissues and organs. For example, in ruminants treated with GH, it causes a large increase in milk production which needs different physiological processes involving the metabolism of nutrients in a number of tissues to be modified (Bauman & Vernon, 1993). This effect of GH on milk production, as well as its increasing growth in animals and decreasing adiposity, has made (recombinant) GH important commercially.

GH affects many different organs within the body, both directly and indirectly. The direct actions of GH are primarily concerned with nutrient partitioning. The indirect

GH effects are exerted *via* IGF's (insulin-like growth factors) produced either locally or released into the circulation by the liver. GH can have acute insulin-like effects on cell metabolism (a minor effect of GH treatment) or chronic effects, promoting linear growth, cellular proliferation and differentiation, along with diabetogenic effects on metabolism.

1.3.2 Effect of growth hormone on lipolysis

As previously stated, it is well established that GH is important not only for linear growth, but also for adipocyte differentiation and energy metabolism in both animals and man. GH treatment of GH-deficient adults reduces the abdominal fat mass and decreases fat cell size (Rosenbaum *et al.*, 1989; Martin *et al.*, 1989). It is not yet fully understood how these effects of GH on adipocyte cells are mediated. Chronic exposure to GH *in vivo* has been shown to increase plasma free fatty acids, to inhibit the conversion of glucose into lipid and to decrease the lipid content of adipose tissue (Goodman & Schwarz, 1974). However, the *in vitro* effects of GH on lipid metabolism, particularly on lipolysis, have been controversial. Pituitary GH was shown to be lipolytic in isolated adipose tissue or adipocytes, but this required the presence of the glucocorticoid, dexamethasone (Fain *et al.*, 1965; Goodman, 1968a). Acute incubation with GH was then shown to potentiate other lipolytic stimuli in rat adipose tissue, such as epinephrine or theophylline (Goodman, 1968b; Goodman, 1968c), suggesting that GH modulated other lipolytic stimuli, rather than acting as an initiator of lipolysis (Goodman & Schwarz, 1974). Highly purified pituitary GH preparations of human GH (hGH) of recombinant DNA origin failed to show lipolytic activity (Frigeri, 1980 ; Frigeri *et al.*, 1982; Bowden *et al.*, 1985), suggesting that contaminants in pituitary GH preparations possessed the lipolytic activity rather than

GH. However, later studies indicated that these same GH preparations were lipolytic *in vitro* in rat adipose tissue in the presence of dexamethasone (Goodman & Grichting, 1983; Goodman, 1984). Pituitary and hGH had comparable lipolytic activity in chicken adipose tissue (Campbell & Scanes, 1985), and were also reported to be lipolytic when administered to hypopituitary children (Van Vliet *et al.*, 1987), showing the importance of the action of GH in overall metabolic regulation in humans as well as showing the intrinsic lipolytic activity of hGH.

Chronic treatment with bGH *in vivo* increases the lipolytic response of adipose tissue to catecholamines *in vitro* in rats (Vernon *et al.*, 1987) and sheep, and *in vivo* in cows (McCutcheon & Bauman, 1986; Peters, 1986; Sechen *et al.*, 1990). *In vitro* studies of chronic GH incubation with sheep adipose tissue explants also increased the response and sensitivity to the β -agonist, isoprenaline, and increased ligand binding to the β -adrenergic receptor (Watt *et al.*, 1991). Other studies (Vernon *et al.*, 1993) suggest that GH enhances lipolysis by increasing the amount of HSL activity associated with the lipid droplet.

However, chronic treatment with GH *in vivo* has no effect on the lipolytic response to catecholamines *in vitro* in bovine adipose tissue (Peters, 1986; Lanna *et al.*, 1992), which raised the possibility that the major effects of GH may be on the antilipolytic system. Chronic treatment with bGH *in vivo* reduces the response to the antilipolytic effects of the adenosine analogue PIA in rat tissue (Doris *et al.*, 1994; Vernon *et al.*, 1987), sheep adipose tissue (Doris *et al.*, 1996) and bovine tissue (Lanna *et al.*, 1992) *in vitro*, while chronic culture of sheep adipose tissue explants with GH decreases the response to both adenosine (PIA) and α_2 -agonists (Vernon *et al.*, 1991b; Doris *et al.*,

1996, 1998). The effect of GH seems to involve a break in the coupling between the inhibitory protein G_i and AC (Doris *et al.*, 1998). Thus, it seems that GH is able to modulate signalling through both the lipolytic and antilipolytic systems.

1.3.3 Effect of growth hormone on lipogenesis

GH can have both insulin-antagonistic and insulin-like effects on lipogenesis. The insulin-like effects are acute and include accelerated transport and metabolism of glucose (Goodman, 1965; Goodman, 1966). The molecular basis of this insulin-like effect has not been fully elucidated, but GH can increase tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) (Souza *et al.*, 1994); a very early step in the insulin signal transduction system (Quon *et al.*, 1994; White & Kahn, 1994). After a period of 2 hours, the insulin-like effects of GH are lost and the chronic, insulin-antagonistic, anti-lipogenic and lipolysis-enhancing effects start to appear (Goodman *et al.*, 1987; Goodman, 1993). However, the insulin-like effect of GH is only seen if the adipose tissue has not been exposed to GH for a period, so the physiological significance is uncertain. This acute insulin-like effect of GH is not seen in sheep adipose tissue (Vernon, 1996).

It has been well documented that GH acts chronically to decrease the rate of lipogenesis, and to antagonise insulin stimulation of lipogenesis in adipose tissue from various species, both *in vivo* and *in vitro* (Nyberg & Smith, 1977; Vernon, 1982; Walton & Etherton, 1986; Etherton *et al.*, 1987). This is due at least in part to growth hormone suppressing the transcription of acetyl CoA carboxylase (ACC) (Bauman & Vernon, 1993) and fatty acid synthase (FAS) (Harris *et al.*, 1993) mRNA. Decreases in the activities of ACC and FAS are also observed following chronic incubation of

adipocytes with GH (Dietz & Schwarz, 1991; Bauman & Vernon, 1993; Goodman, 1993; Vernon *et al.*, 1993). GH also inhibits the activation of ACC by insulin, possibly by inhibiting the synthesis of a protein required for mediation of the insulin-induced activation of the enzyme (Vernon *et al.*, 1991). GH also increases the rate of lipolysis, as described above, and increased lipolysis, if resulting in an accumulation of fatty acids in adipocytes, can lead to decreased lipogenesis (Vernon, 1977). However, the signalling system involved in the chronic, insulin-antagonistic effect of GH on lipogenesis in adipose tissue is not known. Indeed, the system responsible for the insulin induction of lipogenesis itself has not been fully elucidated.

1.3.4 Growth hormone and differentiation

GH exerts a wide variety of actions on cellular differentiation and proliferation either directly or indirectly *via* IGF's, mainly mediated by regulating the transcription of specific genes (Roupas & Herington, 1994). GH promotes differentiation of several pre-adipocyte cell lines, including 3T3-F442A cells and ob 1771 cells (Green *et al.*, 1985; Doglio *et al.*, 1986). GH has been shown to induce insulin-like growth factor-1 (IGF-1) gene expression in ob 1771 pre-adipocytes (Doglio *et al.*, 1987), and to increase the sensitivity of 3T3-F442A cells to the actions of IGF-1 (Zezulak & Green, 1986). IGF-1 cannot substitute for GH in either cell type, however (Nixon & Green, 1984a; Nixon & Green, 1984b; Dani *et al.*, 1990; Catalioto *et al.*, 1992). It has been shown that in 3T3-F442A pre-adipocytes, exposure to GH is essential to differentiation (Yarwood *et al.*, 1998), hence it is perceived as a "commitment factor" (Wabitsch *et al.*, 1995; Hwang *et al.*, 1997; Grégoire *et al.*, 1998). Following exposure to GH, there is an increase in the number of newly differentiated cells by clonal expansion, mediated by the mitotic actions of IGF-1 (Green *et al.*, 1985).

However, GH does not promote the differentiation of all cell types. For example, in the case of 3T3-L1 cells, IGF-1 and insulin are required for differentiation (Smith *et al.*, 1988).

It has been shown that in 3T3-F442A pre-adipocytes, GH also alters the expression of various cytoskeletal proteins (Guller *et al.*, 1992). This may be an explanation for the morphological changes observed in the initial stages of differentiation. GH also stimulates the transcription of early response genes *c-fos* and / or *c-jun* (Doglio *et al.*, 1989; Sumantran *et al.*, 1992), which coincides with transcriptional activation of C/EBP δ and translational activation of C/EBP β . This may contribute to GH's differentiation-promoting effects (Clarkson *et al.*, 1995), although the exact mechanism by which GH exerts its adipogenic effects in pre-adipocytes is still poorly understood.

In contrast to pre-adipocyte cell lines, GH does not appear to be needed for the differentiation of primary pre-adipocytes, for rat or humans (Ailhaud *et al.*, 1992; Wabitsch *et al.*, 1995) or sheep (Soret *et al.*, 1999). Indeed, it inhibits differentiation (Hausman & Martin, 1989; Hausman *et al.*, 1992; Wabitsch *et al.*, 1995, 1996a, 1996b; Soret *et al.*, 1999). GH does stimulate proliferation of human pre-adipocytes (Hausman & Martin, 1989; Gaskins *et al.*, 1990; Wabitsch *et al.*, 1995; Wabitsch *et al.*, 1996a), however, but this effect appears to be induced *via* IGF-1 (Wabitsch *et al.*, 1996a).

1.3.5 The growth hormone receptor

Explanations of the ability of one hormone to direct multiple biological effects have suggested the existence of tissue-specific subtypes of GH receptors (Smal *et al.*, 1987; Press, 1988) or the existence of multiple active domains within the GH molecule (Kostyo, 1986; Salem, 1988). The first stage in GH action involves binding to a specific membrane-associated receptor. GH receptors have been demonstrated on adipocytes from rats to man, confirming that the hormone can act directly on the tissue. The GH receptor has been cloned from several species including man (Leung *et al.*, 1987), rat (Mathews *et al.*, 1989), mouse (Smith *et al.*, 1989), cow (Hauser *et al.*, 1990) and sheep (Adams *et al.*, 1990). The GH receptor (GH-R) appears to be highly conserved in all species where it has been cloned so far, and is a single, membrane-spanning protein of ~624 amino acids. It belongs to the GH / prolactin / cytokine receptor superfamily (Mathews, 1991; Kelly *et al.*, 1992), and has three domains (Waters *et al.*, 1990);

- (1) an extracellular domain of ~250 amino acids, which is very similar to the GH-binding protein of plasma,
- (2) a short trans-membrane domain of ~25 amino acids, and
- (3) an intracellular domain of ~350 amino acids which lacks intrinsic tyrosine kinase domains.

The number of receptors varies with depot, sex and age (Vernon & Flint, 1989).

A very early step in GH-R signalling is the growth hormone-induced sequestration and activation of a GH-R tyrosine kinase, JAK-2 (Argetsinger *et al.*, 1993). GH binding has also been shown to promote dimerisation of the GH-R extracellular domain (Cunningham *et al.*, 1991; de Vos *et al.*, 1992); altered forms of GH that are unable to

cause this dimerisation are also incapable of eliciting signalling through the full-length cell surface GH-R (Ishizaka-Ikeda *et al.*, 1993; Silva *et al.*, 1993). Additionally, a disulphide-linked form of the GH-R can be detected within seconds of GH binding, and GH-R's that are precipitable by phosphotyrosine antibodies in response to human GH are enriched in this disulphide-linked form (Frank *et al.*, 1994). These findings all indicate that GH initiates its signal cascade by activating a protein complex including at least a GH-R dimer and JAK2 in a mixture of covalent and non-covalent associations.

The GH-R protein exists in two molecular mass forms ; the full length receptor and a smaller soluble GH binding protein (GHBP), which circulates in the blood and binds to GH, and is homologous to the extracellular domain of the GH-R. There have been two mechanisms proposed for the production of the GHBP ; (1) it is due to alternative splicing of a single primary transcript, and (2) it is due to specific proteolysis of the membrane receptor. In most species, as only a single mRNA transcript (~4.5 kb) has been identified by Northern Blot analysis, the second mechanism has been suggested to explain the production of GHBP. The biological functions of this GHBP remain to be clarified. It could act as a reservoir for GH in the circulation. Decreased degradation and metabolic clearance of GH has been reported in a rat model when GH is bound to the GHBP (Baumann *et al.*, 1988). Alternatively, the binding protein could serve to block GH actions, preventing further binding to membrane receptors (Lim *et al.*, 1990).

1.3.6 Growth hormone signalling

The GH induced intracellular signal pathway is not yet fully understood. As stated above, it has been shown both by mutational analysis and by X-ray crystallography that a GH molecule binds to two receptor molecules (Cunningham *et al.*, 1991; De Vos *et al.*, 1992), and that hormone-mediated receptor dimerisation is necessary for generation of an activated receptor (Fuh *et al.*, 1992). The GH-R may be internalised following binding of the ligand, but the role of internalisation in signal transduction, if any, is unknown (Roupas & Herington, 1989). Even though the receptor lacks an intrinsic tyrosine kinase domain, tyrosine phosphorylation of several proteins in response to GH (Tornqvist *et al.*, 1991 ; Anderson, 1992; Campbell *et al.*, 1993), including the GH-R complex (Carter-Su *et al.*, 1989) has been reported. Phosphorylation of other cellular proteins may therefore be one mechanism by which GH transmits an intracellular signal (Wang *et al.*, 1993).

It has been shown that the GH-R in cells associates with a separate tyrosine kinase, called Janus tyrosine kinase-2 (JAK-2), which is a member of cytosolic tyrosine kinases. This kinase was initially shown in 3T3-F442A cells to be able to associate with the occupied GH-R, resulting in activation of JAK2 kinase and tyrosine phosphorylation of the GH-R (Argetsinger *et al.*, 1993). JAK2, and the other members of the Janus tyrosine kinase family, has two kinase-like domains (Ihle *et al.*, 1994). Studies using mutated GH-R showed that the proline-rich Box 1 motif of the cytoplasmic domain is primarily required for the association of JAK2 with GH-R and GH-dependent activation of JAK2 (Vanderkuur *et al.*, 1994). However, the N-terminal quarter of the cytoplasmic domain of the GH-R appears to augment the interaction with JAK2. It has been suggested using mutational studies (Vanderkuur *et*

al., 1994) that at least one of the tyrosyl residues present in the N-terminal half of the cytoplasmic domain of the GH-R is phosphorylated by JAK2. JAK2 has also been shown to associate with the erythropoietin receptor (Witthuhn *et al.*, 1993), interleukin-3 receptor (Silvennoinen *et al.*, 1993) and the prolactin receptor (Lebrun *et al.*, 1994).

It has been revealed using pre-adipocyte cell lines that at least three pathways are involved in the intracellular signalling cascades activated by GH ; activation of mitogen-activated protein (MAP) kinase and p90^{rk} (Moller *et al.*, 1992; Anderson, 1992) ; activation of p70^{S6K} (Anderson, 1993) ; and activation and translocation of STAT-1 (signal transducers and activators of transcription) (Roupas & Herington, 1994; Waters *et al.*, 1994; Ihle & Kerr, 1995), but whether these systems are involved in the chronic metabolic effects of GH in mature adipocytes or not is unknown.

STAT proteins are a family of cytosolic transcription factors, which are the products of multiple genes and alternative splicing. These are phosphorylated by members of the JAK family of tyrosine kinases (see Lamb *et al.*, 1996). STAT proteins undergo tyrosine phosphorylation in the cytoplasm, followed by translocation to the nucleus where variations in the complexes formed with other transcription factors confer specificity of interaction with different DNA response elements (Hunter *et al.*, 1993). GH phosphorylates and causes nuclear accumulation of STAT1 α (Kilgour & Anderson, 1994; Meyer *et al.*, 1994). Phosphorylation of STAT1 by GH results in the formation of complexes that can interact with the *sis*-inducible element (SIE) of *c-fos* (Meyer *et al.*, 1994). STAT1 is a subunit of the ISGF3 complex, which comprises a 48-kDa DNA-binding component, an 84/91-kDa protein and a 113-kDa protein, termed p48, p84, p91 and p113, respectively. These protein subunits of ISGF3 are

localised in the cytoplasm of unstimulated cells. Formation of the complex and its migration to the nucleus requires tyrosine phosphorylation of p91/p84 and p113, which is mediated by JAK. It has also been suggested that GH can activate STAT3/APRF (acute-phase response factor) and STAT5/MGF (mammary gland factor) in a cell-specific manner (Meyer *et al.*, 1994; Gronowski & Rotwein, 1994; Campbell *et al.*, 1995; Gouilleux *et al.*, 1995; Thomas *et al.*, 1995; Leaman *et al.*, 1996; Yi *et al.*, 1996).

MAP kinases are expressed in all cells, and can be activated by hormones, growth factors, neurotransmitters and other cell stimuli (Anderson, 1992b). The activation of MAP kinase requires phosphorylation upon both tyrosine and threonine residues (Anderson *et al.*, 1990; Gomez & Cohen, 1991) by dual specificity MAP kinase kinases, or MEKs (Nakielnny *et al.*, 1992; Crews & Erikson, 1992). It has been shown that GH induces rapid and transient activation of p44 and p42 MAP kinases and p90^{rsk} in 3T3-F442A pre-adipocytes (Anderson, 1992b). To be able to activate the MAP kinases, GH seems to require the presence of JAK2, *Ras* and *Raf* (Winston & Hunter, 1995). The S6 kinase, p90^{rsk}, lies downstream of the p42 and p44 MAP kinases, and it has been suggested that protein kinase C (PKC) plays a role in the GH-dependent activation of MAP kinases and p90^{rsk} (Anderson *et al.*, 1992a).

GH also phosphorylates and activates the S6 ribosomal kinase p70^{S6K} in 3T3-F442A cells (Anderson, 1993), which is involved in the regulation of translation *via* phosphorylation of the S6 ribosomal proteins. Activation of p70^{S6K} is *via* a MAP kinase independent pathway and is thought to involve phosphatidylinositol 3-kinase

(PI-3Kinase). Certain second messenger molecules also increase in response to GH such as diacylglycerol (DAG) (Catalioto *et al.*, 1990).

The studies described above relate to GH signalling in its role as a commitment factor for differentiation of 3T3-F442A cells, and to a lesser extent, the acute, insulin-like effects of GH. The involvement of these various signalling pathways in the chronic effects of GH is not fully resolved, but studies with sheep adipocytes using inhibitors such as rapamycin and PD98059 suggest that neither p70^{S6K} nor MAP kinase are involved with chronic related effects of GH (see section 1.3.3). However, anti-lipogenic, insulin-antagonistic effects of GH appear to involve both protein serine kinases and phosphatases (Vernon, 1996) by use of the inhibitors H7, staurosporine and okadaic acid. Chronic exposure to phorbol ester partly attenuated the antilipogenic effect of GH, suggesting the involvement of one or more isoforms of PKC (Vernon, 1996). Studies carried out by Borland *et al.* (1994) showed that the inhibition of lipogenesis in sheep adipose tissue by GH required polyamines. The effects of polyamines include a role in gene transcription (Scalabrino *et al.*, 1991; Sjöholm, 1993), activation of a number of protein kinases and phosphatases (Morgan, 1990) and enhancing hormone binding to plasma membrane receptors (Pedersen *et al.*, 1989). Studies using actinomycin D, an inhibitor of gene transcription, implicated a protein with a half-life of less than 3 hours in the inhibition of lipogenesis by GH (Borland *et al.*, 1994).

1.4 Tumour necrosis factor alpha

The cytokine family is a large group of polypeptides which includes interleukins (IL) 1-10, tumour necrosis factors (TNF) (α and β) and interferons. They have numerous and diverse actions on many cell types, but are most noted for their effects on cells of the immune system (Hopkins, 1990; Dinarello, 1990).

The TNF “family” includes two structurally and functionally related proteins, TNF α or cachetin (Carswell *et al.*, 1975; Beutler *et al.*, 1985) and TNF β or lymphotoxin (Williams & Granger, 1968; Ruddle & Waksman, 1968). TNF α is produced mainly by neutrophils, activated lymphocytes, macrophages, natural killer cells, astrocytes, endothelial cells, smooth muscle cells, adipocytes and some transformed cells, whereas TNF β is a product of lymphoid cells (Vilcek & Lee, 1991). There is around 30% amino acid residue homology between them, and they bind to the same cell surface receptors, producing a vast range of similar, but not identical, effects. These effects include the ability to kill certain tumour cells directly, from which their name derives. The TNFs have a central role in initiating (together with IL-1) the cascade of other cytokines and factors that make up the immune system’s response to infection and sometimes to cancer. This response is critical in the successful resolution of infectious and metastatic diseases, but can occur in an uncontrolled manner, thereby damaging the host. The most dramatic example of this is septic shock following infection by Gram-negative bacteria (Tracey *et al.*, 1987), and administration of TNF α or TNF β at sufficiently high levels can reproduce virtually all of the symptoms of septic shock in otherwise healthy animals and humans. Over-production of TNF has been implicated as playing a role in a number of pathological conditions, including

cachexia (progressive wasting) (Beutler *et al.*, 1985; Oliff, 1988), autoimmune disorders (Pujol-Borrell *et al.*, 1987) and meningococcal septicemia (Waage *et al.*, 1987).

Mature human TNF α is a polypeptide of 157 amino acid residues, with mouse, rat and rabbit TNF α having one amino acid less (Vilcek & Lee, 1991). Human TNF β has a higher molecular mass than TNF α (25-kDa and 17-kDa, respectively) under denaturing conditions due to extra amino acid residues and from N-glycosylation (Aggarwal *et al.*, 1984; Aggarwal *et al.*, 1985). Human TNF α shows no N-glycosylation, but murine TNF α is N-glycosylated (Vilcek & Lee, 1991). The biologically active native forms of both TNFs are non-covalently linked trimers (Jones *et al.*, 1989; Eck & Sprang, 1989; Eck *et al.*, 1992). TNF α occurs as a secreted, soluble form and as a membrane-anchored form. TNF α has a long precursor sequence with both hydrophilic and hydrophobic domains, and apparently occurs as a membrane-bound form from which the soluble 17-kDa factor is derived by cleavage of the extracellular domain (Kriegler *et al.*, 1988; Luettig *et al.*, 1989; Perez *et al.*, 1990). Current evidence suggests that the membrane-anchored form of TNF α that exists on the surface of macrophages and / or monocytes, in addition to serving as a reservoir for release of soluble TNF α , has cytotoxic activity (Kriegler *et al.*, 1988; Luettig *et al.*, 1989; Perez *et al.*, 1990), and may also play an important role in intercellular communication (Aversa *et al.*, 1993; Macchia *et al.*, 1993).

1.4.1 Tumour necrosis factor alpha and adipose tissue

Fatty acids are essential to lymphocyte function, both as fuel and as precursors for the synthesis of lipid-based messenger molecules (Calder, 1995). Nearly all major lymph nodes are embedded in adipose tissue (Yoffey & Courtice, 1970) and most peripheral adipose depots contain one or more lymph nodes (Pond, 1996). Adipocytes have recently been found to share a range of signal molecules with lymphoid cells, including macrophage colony-stimulating factor (Levine *et al.*, 1998) and TNF α (Argiles *et al.*, 1997). The two types of TNF α receptors have been found on adipocytes. Type II receptors to TNF α are present continuously on adipocytes around the popliteal lymph node, and type I receptors increase on these cells within 30 minutes of lipopolysaccharide (a known elicitor of TNF α (Old, 1985)) being injected into a rat's lower leg, and on those around other nodes within 24 hours (MacQueen & Pond, 1998). These experiments reveal that the capacity of adipocytes to respond to this cytokine is prompted by immune stimulation, and can be highly localised to the perinodal adipocytes. This is true in the case of guinea pigs and rats, however its occurrence in other species is uncertain.

It has been shown that lipolysis in adipose tissue explants from around major lymph nodes increased up to 3-fold when incubated for 48 hours with mixtures of macrophages and lymphocytes with or without a mitogen (Pond & Mattacks, 1995). However, the same conditions produced minimal change in the glycerol released from explants from the perirenal depot, which lacks lymph nodes, and only small increases in samples taken from sites remote from lymph nodes in other depots. This indicates that the site-specific properties of adipose tissue around the lymph nodes equip the

tissue to nourish or regulate (or both) the metabolism of the lymphoid cells in the nodes. Adaptive local interactions with peripheral lymph nodes might be a major reason for the evolution among mammals of the partitioning of adipose tissue into so many small depots (Pond, 1978).

Recent studies have shown that TNF α can play an important role in obesity and diabetes in addition to its well-known role in cachexia (Hotamisligil & Spiegelman, 1994; Hotamisligil *et al.*, 1994; Hotamisligil *et al.*, 1995). TNF α , which is synthesised by many cell types, including adipose cells, is a pleiotropic factor that exerts a variety of effects on adipocytes, including thermometabolism, lipogenesis and lipolysis. Collectively, these metabolic effects modulate body fat mass. It has also recently been reported that TNF α can directly induce adipocyte apoptosis in cell culture (Qian *et al.*, 1998; Porras *et al.*, 1997; Prins *et al.*, 1997). Qian *et al.* (1998) have recently shown that TNF α plays a role in leptin-induced lipolysis in adipocytes, and that leptin, a protein whose synthesis is localised in adipose tissue (Zhang *et al.*, 1994) and which reduces fat depot size by reducing food intake and increasing energy expenditure (Halaas *et al.*, 1997), regulates TNF expression in adipose tissues.

TNF α causes a decrease in the activity of lipoprotein lipase (LPL) (Fried & Zechner, 1989; Grünfeld *et al.*, 1989), a decrease in the expression of the glucose transporter GLUT4 (Stephens & Pekala, 1991) and an increase in HSL (Patton *et al.*, 1986). The regulation of these and other genes could affect the adipocyte insulin sensitivity and lipid accumulation. Therefore, the production of TNF α by adipose tissue could be a local regulator of fat size, and the overproduction of TNF α in adipocytes of obese

animals could represent a form of "adipostat", *i.e.*, a normal homeostatic mechanism designed to limit adipocyte size in the face of overconsumption (Spiegelman *et al.*, 1993; Spiegelman & Hotamisligil, 1993).

1.4.2 Tumour necrosis factor alpha and lipid metabolism

TNF α has a wide variety of effects on lipid metabolism. It decreases the activity of LPL in adipose tissue both *in vivo* (Semb *et al.*, 1987; Evans & Williamson, 1988) and *in vitro* (Price *et al.*, 1986; Kawakami *et al.*, 1987). LPL is secreted by adipocytes and is one of the key enzymes involved in lipogenesis. TNF α also stimulates hepatic lipid synthesis (Feingold & Grünfeld, 1987; Feingold *et al.*, 1989), which may be the main cause for hypertriglyceridaemia that follows TNF administration (Chajek-Shaul *et al.*, 1989; Grünfeld & Feingold, 1991), and increases glucose uptake by peripheral tissues (Evans *et al.*, 1989b), hepatic uptake of amino acids (Warren *et al.*, 1987; Argiles *et al.*, 1989; Argiles & Lopez-Soriano, 1990), and skeletal muscle proteolysis (Argiles *et al.*, 1992; Llovera *et al.*, 1993). TNF and interleukin-6 also mobilise fatty acids from the periphery, which are re-esterified into triglyceride in the liver (Feingold & Staprans, 1990; Nonogaki *et al.*, 1995). The net result is increased secretion of very low density proteins (VLDL) by the liver, resulting in a sustained increase in triglyceride levels (Feingold & Grünfeld, 1987; Feingold *et al.*, 1991, 1992). TNF α and interleukin-1 have both been shown to inhibit glucose transport in adipocytes (Hauner *et al.*, 1995), and consequently to decrease the availability of substrates for lipogenesis. No direct action of TNF α has been shown on *de novo* lipogenesis in adipose tissue of starved rats (Feingold & Grünfeld, 1987), however TNF α has been shown to decrease ACC (a key lipogenic enzyme) during pre-adipocyte differentiation

by a decrease in its mRNA (Pape & Kim, 1988). Disorders in lipid metabolism like hypertriglyceridemia are observed as a consequence of infection, and cachexia, manifested by loss of muscle and so fat, is observed in man under conditions in which circulating TNF α can be measured.

1.4.3 Tumour necrosis factor alpha and lipolysis

There have been conflicting reports published on the effects of TNF α on the lipolytic system in primary adipocytes and established cell lines. In 1987, Rofo and colleagues found that TNF α did not stimulate basal lipolysis in isolated rat adipocytes, although TNF α did increase the response to adrenaline after 1 hour incubation (Rofo *et al.*, 1987). However, it has been reported by Green *et al.* (1994) that TNF α chronically caused an increase in basal lipolysis in rat epididymal adipocytes, with the effects of TNF α only being seen after 6 hours of incubation. However, no change in either the response to isoproterenol or expression of HSL was observed (Green *et al.*, 1994). Very recent studies by Green show that TNF α appears to stimulate basal lipolysis in rat adipocytes by decreasing the inhibitory G-protein, G_i, concentrations, thus blocking the inhibitory action of endogenous adenosine (Gasic *et al.*, 1999). Chronic exposure to TNF α has also been shown to increase basal lipolysis in newly-differentiated human adipocytes in cell culture (Hauner *et al.*, 1995); as with the studies by Green *et al.* (1994), TNF α was required to be present for over 6 hours for any effects to be seen on the lipolytic rate.

Studies using differentiated cells from the clonal cell line 3T3-F442A also showed that TNF α increased basal lipolysis after an incubation period of 8 hours (Hardardottir

et al., 1992; Feingold *et al.*, 1992). These studies found that TNF α appeared to stimulate prostaglandin (PG) synthesis (Hardardottir *et al.*, 1992) and that the presence of indomethacin (an inhibitor of PG synthesis) prevented the increase in basal lipolysis induced by TNF α (Feingold *et al.*, 1992), suggesting that the lipolytic actions of TNF α are mediated by PG. Indomethacin can also act as a PPAR γ agonist (Lehman *et al.*, 1997), which could account, in part at least, for the findings (Feingold *et al.*, 1992) described above. However, PGE₂ is a potent inhibitor of basal and hormone-induced lipolysis in adipocytes (Kather, 1981; Richelsen *et al.*, 1984; Richelsen, 1987), which contradicts these findings.

Other studies using 3T3-L1 adipocytes have shown that TNF α chronically (minimum of 6 hours incubation) increases basal lipolysis (Souza *et al.*, 1998a). These studies show that the PPAR γ agonist, a thiazolidinedione (TZD) rosiglitazone (BRL49653), reduces the ability of TNF α to stimulate basal lipolysis in 3T3-L1 adipocytes; rosiglitazone had no effect on basal lipolysis or isoproterenol-stimulated lipolysis in the absence of TNF α (Souza *et al.*, 1998a). This may be one of the mechanisms which enables TZDs to increase systematic insulin sensitivity. TNF α was also found to cause a large decrease in the expression of perilipin A and a smaller reduction in the expression of HSL; an effect which was partially blocked by rosiglitazone (Souza *et al.*, 1998a). Overexpression of perilipin resulted in prevention of TNF α to increase lipolysis in the 3T3-L1 adipocytes, but had no effect on isoproterenol-stimulated lipolysis or on isoproterenol-induced migration of perilipins from the lipid droplet (Souza *et al.*, 1998b). This suggests that TNF α regulates lipolysis, in part at least, by decreasing perilipin protein levels at the lipid droplet surface (Souza *et al.*, 1998b).

The reasons for why TNF α appears to use different mechanisms for modulation of lipolysis in the different systems used is not clear. What is apparent is that TNF α increases basal lipolysis, but only after several hours of incubation, although the length of time varies between the systems. Green *et al.* (1994) found no effect of TNF α on HSL levels in rat adipocytes, but Souza *et al.* (1998a) found that incubation with TNF α results in a decrease in HSL levels in 3T3-L1 adipocytes, although the effect was small. However, Green did not investigate the effect of TNF α on perilipin, and the effects of TNF α on isoproterenol-stimulated lipolysis were not looked at by Souza. Therefore their results may not be inconsistent. However, the studies by Feingold *et al.* (1992) conflict with those of Green (Green *et al.*, 1994), as PG's inhibit lipolysis and TNF α stimulates lipolysis.

1.4.4 Tumour necrosis factor alpha and insulin resistance

TNF α is also an important mediator of insulin resistance in obesity and diabetes through its ability to inhibit the tyrosine kinase activity of the insulin receptor (IR), and tyrosine phosphorylation of one of its substrates, insulin receptor substrate-1 (IRS-1). This effect is observed in various cell lines such as adipocytes, fibroblasts, hepatocytes and myeloid cells (Feinstein *et al.*, 1993; Hotamisligil *et al.*, 1994b; Peraldi *et al.*, 1996). Treatment of cultured murine adipocytes with TNF α was shown to induce serine phosphorylation of IRS-1 (Kanety *et al.*, 1995; Hotamisligil *et al.*, 1996) and convert IRS-1 into an inhibitor of the IR tyrosine kinase activity *in vitro* and in intact cells (Hotamisligil *et al.*, 1996). This effect is dependent upon the phosphorylation of IRS-1 and is reversible by dephosphorylation of IRS-1 by alkaline phosphatase. This mechanism appears to be the one by which TNF α induces insulin

resistance in animals. TNF α is expressed at a higher level in the adipose tissue from genetically obese rodents (Hotamisligil *et al.*, 1993; Hofmann *et al.*, 1994). After infusion of anti-TNF α binding protein, insulin sensitivity improves (Hotamisligil *et al.*, 1993), owing to improved IR tyrosine kinase activity (Hotamisligil *et al.*, 1994). This indicates that overexpression of TNF α in adipocytes is in part responsible for insulin resistance in animal (Hotamisligil *et al.*, 1993; Hofmann *et al.*, 1994; Hamann *et al.*, 1995) and human obesity (Hotamisligil *et al.*, 1995; Kern *et al.*, 1995).

1.4.5 Tumour necrosis factor alpha receptor

Tumour necrosis factor receptors (TNF-R) have been detected on a wide variety of normal tissues and cell lines sensitive or resistant to TNF α (Kull *et al.*, 1985; Baglioni *et al.*, 1985; Tsujimoto *et al.*, 1985; Creasey *et al.*, 1987). There have been two immunologically distinct TNF receptors identified ; 55kDa (Loetscher *et al.*, 1990; Schall *et al.*, 1990) and 75kDa (Smith *et al.*, 1990; Dembic *et al.*, 1990), each of which binds TNF α and TNF β with a similar affinity (Loetscher *et al.*, 1990; Smith *et al.*, 1990). Both receptors have clear sequence similarity in their extracellular domains, where they both contain a characteristic 6-cysteine consensus motif repeated four times. In contrast, their cytoplasmic region sequences are entirely unrelated, suggesting different modes of signalling and function (Dembic *et al.*, 1990; Loetscher *et al.*, 1990; Schall *et al.*, 1990; Smith *et al.*, 1990). The receptors are glycoproteins with a single membrane-spanning hydrophobic segment. Based mostly on their similarities in their extracellular domains, these receptors belong to a receptor superfamily which includes the low-affinity nerve growth factor receptor and the FAS-antigen or APO-1 (Nagata, 1993; Smith *et al.*, 1994). Most of the known TNF

responses occur by the activation of p55 TNF-R (Wiegmann *et al.*, 1992), and this has been confirmed *in vivo*. Mice deficient for the p55 TNF-R were resistant to lipopolysaccharide or toxic shock, yet proved compromised in their ability to clear intracellular bacteria like *Listeria monocytogenes* (Pfeffer *et al.*, 1993; Rothe *et al.*, 1993). However, thymocyte proliferation is associated with the p75 TNF-R (Tartaglia *et al.*, 1991), and cytotoxicity may be a function of p75 TNF-R acting alone or together with p55 TNF-R (Heller *et al.*, 1992; Grell *et al.*, 1993). There is no evidence that the TNF receptors possess intrinsic tyrosine kinase activity, unlike some major growth factor receptors. A comparison of the intracellular sequences of the two TNF-R's shows no obvious sequence similarity to known tyrosine- or serine/threonine-kinase encoding sequences. Both receptors are rich in serine and threonine residues, which may serve as sites of phosphorylation by other cellular kinases. The p55 TNF-R also contains a tyrosine residue, a possible phosphorylation site for heterologous tyrosine kinase.

In addition to the receptors and ligands, two soluble TNF inhibitory proteins have been discovered and shown to be truncated extracellular regions of the two receptors (Olsson *et al.*, 1989; Engelmann *et al.*, 1990; Seckinger *et al.*, 1990).

The active form of both TNF α and TNF β appears to be the trimeric molecule. Both factors crystallise in the trimeric form (Hakoshima & Tomita, 1988; Jones *et al.*, 1989; Eck & Sprang, 1989; Eck *et al.*, 1992). Trimeric TNF α and TNF β have also been detected in solution, and recombinant TNF α and TNF β were found to combine in solution with soluble TNF receptors to form complexes in which three receptor

molecules bind to one TNF α or TNF β trimer (Loetscher *et al.*, 1991b; Schoenfeld *et al.*, 1991). It has been suggested that one receptor interacts with sites on two adjacent subunits, and that the TNF trimer thus has three spatially distinct but equivalent receptor-binding sites.

1.4.6 Tumour necrosis factor alpha and signal transduction

Phosphorylation of several distinct proteins has been shown to occur within minutes after the exposure of cells to TNF (Schutze *et al.*, 1989; Robaye *et al.*, 1989; Marino *et al.*, 1989; Kronke *et al.*, 1991), which is probably due to the activation of several major cellular kinases. Evidence suggests that TNF α and interleukin-1 β employ the sphingomyelin pathway to effect signal transduction by their receptors. This pathway is initiated by hydrolysis of a neutral plasma membrane sphingomyelin to ceramide by the action of sphingomyelinase (SMase) (Kim *et al.*, 1991). Ceramide serves as a second messenger, stimulating a serine/threonine membrane-bound 97-kDa ceramide-activated protein kinase to transduce the cytokine signal (Liu *et al.*, 1994), in part through mitogen-activated protein (MAP) kinases and transcription factors such as nuclear factor (NF)- κ B and activating protein AP-1. NF- κ B and AP-1 activation by TNF depends upon the activities of *Ras* and *Raf* (Finco & Baldwin, 1993), and *fos* and *jun* (Brenner *et al.*, 1989), respectively. These factors mediate the induction of many proteins central to inflammatory processes and immune responses, such as cytokines, cell-adhesion molecules, growth factors, metalloproteinases, and other proteins that participate in the production of PG's, leukotrienes and nitrogen oxide (Angel & Karin, 1991; Baldwin, 1996). TNF reportedly also activates the 42- and 44-kDa MAP kinases and increases their tyrosine phosphorylation, as well as their ability to

phosphorylate the standard substrate myelin basic protein (MBP) (Vietor *et al.*, 1993). The phosphorylation and activation of the 42-kDa MAP kinase by TNF parallels an increase in cellular sphingomyelin hydrolysis and the addition of both bacterial SMase or cell-permeable ceramide analogues activate the p42 MAP kinase in a time-dependent manner, similar to TNF (Raines *et al.*, 1993). Therefore, both the ceramide-activated kinase and the MAP kinases are activated by TNF and ceramides. Activation of MAP kinases leads to distinct cellular responses mediated by phosphorylation of specific target substrates (Marshall, 1994).

Arachadonic acid (AA) release by TNF occurs in a number of cell lines, mediating increases in both the secreted 14-kDa (Oka & Arita, 1991) and the cellular 85-kDa (Hoeck *et al.*, 1993) phospholipase (PL) A₂, which are enzymes known for the production of AA by cells. cPLA₂ is selective for AA (Lin *et al.*, 1992), and provides a source for the production of biologically active lipid mediators such as PG's and leukotrienes. TNF alone induces phosphorylation of cPLA₂, but when calcium is present there is a marked increase in cPLA₂ activity. In combination with calcium-mobilising agents at sites of inflammation, the released AA could serve both a regulatory function and have pro-inflammatory action. cPLA₂ is phosphorylated on serine residues by PKC and the p42 MAP kinase, and the MAP kinase-mediated phosphorylation is essential for receptor-induced cPLA₂ activation (Lin *et al.*, 1993; Nemenhoff *et al.*, 1993). Growth factors induce MAP kinase activity through a signal cascade of signal transfer reactions including *ras* and *raf* proteins, and the serine/threonine MAP kinase kinases (Thomas, 1992). Since the TNF / SMase initiated protein phosphorylation cascade stimulates a MAP kinase pathway and the p42 MAP kinases activate cPLA₂, it is conceivable that the ceramide-activated protein

kinase is an intermediate in the MAP kinase action pathway, particularly if *c-Raf* could serve as a substrate for ceramide-activated protein kinase (Liu *et al.*, 1994). It has also been reported that the p38 MAP kinase pathway is activated in TNF-treated cells, which has a crucial but selective role in gene induction, such as synthesis of interleukin-6 and granulocyte macrophage colony-stimulating factor. (Beyaert *et al.*, 1996).

Within minutes after binding to the p55 TNF-R, TNF stimulates the production of diacylglycerol (DAG) from membrane phospholipids by activation of phospholipase C (PLC). Phosphatidylcholine (PC) is hydrolysed by phosphatidylcholine-specific PLC (PC-PLC), which results in DAG generation. The DAG generated by PC-PLC appears central to the activation of at least two other signalling enzymes, protein kinase C (PKC) (Brenner *et al.*, 1989; Kronke *et al.*, 1991) and acidic SMase. The activation of PKC by this pathway is probable as a release of Ca^{2+} from internal stores, an increase in PLD activity, and an increase in inositol 1,4,5-triphosphate levels have not been seen after TNF treatment. PKC mediates a number of TNF actions, including the induction of *jun* and *fos* proteins that are components of the AP-1 transcription factor (Brenner *et al.*, 1989). There is also evidence for a rapid increase in cyclic AMP levels and resulting PKA activation (Zhang *et al.*, 1988). The activation of the acidic SMase, C-type phospholipase, causes a breakdown of sphingomyelin to produce ceramides. Although the presence of phorbol esters causes downregulation of PKC, they do not alter the SMase activity, indicating that the action of DAGs on SMase is independent of its effects on PKC (Kolesnick, 1987). The ceramide produced from the breakdown of sphingomyelin acts as a second messenger, mediating a number of cellular responses (Hannum, 1994). While ceramide produced

by neutral SMase at or within the plasma membrane seems to trigger a plasma membrane-associated kinase (Liu *et al.*, 1994), ceramide produced by the acidic SMase is associated with the endolysosomal compartments and therefore may have different consequences.

Another class of enzymes that are strongly activated by TNF are the stress-activated protein kinases (SAPK; Kyriakis *et al.*, 1994) or *c-jun* N-terminal kinases (JNK; Dérijard *et al.*, 1994). SAPK/JNK are potentially induced by environmental stresses, such as inhibitors of protein synthesis, inflammatory cytokines, changes in osmolarity, heat shock and UV irradiation (Kyriakis *et al.*, 1994; Dérijard *et al.*, 1994), and have been implicated in the induction of programmed cell death (Xia *et al.*, 1995; Santana *et al.*, 1996; Verheij *et al.*, 1996). However, several studies have recently demonstrated that SAPK/JNK activation by p55 TNF-R is mediated through the protein, TNF-R-associated factor 2 (TRAF2), and that it occurs in a non-cytotoxic manner (Liu *et al.*, 1996; Natoli *et al.*, 1997; Reinhard *et al.*, 1997). Additional studies are required to fully elucidate the function of SAPK/JNK in the signal transduction pathway initiated by p55 TNF-R.

1.5 Insulin

The insulin molecule consists of two polypeptide chains, A (21 amino acids) and B (30 amino acids), connected by two disulphide bridges. This two-chain structure has been present throughout evolution, but major variations in the sequence are observed between species. Insulin is formed from a biosynthetic precursor of a higher molecular weight called proinsulin, which originates in the pancreas.

Insulin is among the most potent of anabolic agents, promoting the synthesis and storage of carbohydrates, lipids and proteins, and inhibiting their degradation and release into the circulation. The physiological actions of insulin are characterised by a wide variety of cellular effects, including modulation of glucose and amino acid transport, activities of key enzymes in intermediary metabolism, rates of protein DNA and RNA synthesis, transcription of specific genes, and cellular growth and differentiation; regulation of lipid metabolism in adipose tissue by insulin is described in section 1.2.5. It is very unlikely that a single mechanism accounts for all the actions of insulin, and therefore different biochemical pathways are required for the regulation of cellular metabolism and growth.

1.5.1 The insulin receptor

The insulin receptor is present in virtually all vertebrate tissues, although the concentration varies from as few as 40 receptors on circulating erythrocytes to more than 200,000 receptors on adipocytes and hepatocytes. The receptor gene is located on the short arm of human chromosome 19, is more than 150 kilobases in length, and contains 22 exons which encode a 4.2-kb cDNA (Seino *et al.*, 1990). The insulin receptor (IR) is a heterotetrameric transmembrane glycoprotein comprised of two extracellular α subunits and two transmembrane β subunits linked by disulphide bonds. The α subunits contain the insulin-binding domain, while the transmembrane β subunits function as a tyrosine-specific protein kinase that undergoes autophosphorylation following insulin binding (for reviews, see White & Kahn, 1994; Cheatham & Kahn, 1995). Three tyrosine residues in the tyrosine kinase domain of the receptor (1158, 1162 and 1163) are phosphorylated *in trans* by the reciprocal half-receptor (Lammers *et al.*, 1990). To propagate the signal, the autophosphorylated

receptor binds directly to proteins containing Src homology domains (SH2 proteins). The binding of SH2 proteins depends on the amino acid sequence surrounding the tyrosine autophosphorylation site in the receptor (Cantley *et al.*, 1991). Phosphorylation of all three tyrosines appears to be necessary to allow full activation of the receptor's tyrosine kinase activity towards other substrates, and to allow normal signalling to downstream effectors (Murukami & Rosen, 1991). Autophosphorylation activates the IR kinase (Zick *et al.*, 1983) and enables it to phosphorylate endogenous proteins, including IR substrate-1 (IRS-1) (Sun *et al.*, 1991), IRS-2 (Sun *et al.*, 1995) and Shc (Pronk *et al.*, 1993). These, in turn, are linked to downstream signal transduction molecules, eventually culminating in cellular biological responses (White & Kahn, 1994; Cheatham & Kahn, 1995; Saltiel, 1996; Waters & Pessin, 1996).

The unoccupied α -subunit of the IR inhibits the tyrosine kinase activity of the β -subunit. Removal of the α -subunits by proteolytic cleavage or deletion mutagenesis, or certain point mutations in the α -subunit, relieve this inhibition. Insulin and epidermal growth factor (EGF) receptors may use similar mechanisms for ligand regulation, since the IR kinase domain (intracellular) linked to an EGF binding domain (extracellular) *via* the transmembrane domain is stimulated by EGF (Riedel *et al.*, 1989). The external ligand binding domain of the IR is linked to the tyrosine kinase by a single transmembrane segment, which has a broad tolerance for structural changes or substitutions (Frattali *et al.*, 1991; Ihle *et al.*, 1990). However, the IR is constitutively activated by substitution of the transmembrane segment from the oncogene *v-erbB-2*, which contains Val⁶⁶⁴→Glu mutation (Cheatham *et al.*, 1993); an

analogous point mutation (Val⁹³⁸→Asp) in the transmembrane segment of the IR also partially activates the receptor kinase (Longo *et al.*, 1992).

Proteins known to associate with IRS-1 are, phosphatidylinositol 3-kinase (the lipid kinase ; PI-3Kinase), *Grb2* (an adapter protein which associates with the guanine nucleotide exchange factor Sos), *syp* (a phosphotyrosine phosphatase) and *nck* (a linker protein) (Skolnik *et al.*, 1993; Tobe *et al.*, 1993; Kuhne *et al.*, 1993; Lee *et al.*, 1993).

1.5.2 Insulin and signal transduction via PI-3kinase

Unlike many other receptor tyrosine kinases, the phosphorylated tyrosine residues on the receptor itself do not appear to act primarily as docking sites for *Src* homology 2 domain-containing signal transduction proteins. The insulin and insulin-like growth factor-1 receptors phosphorylate one or more substrate proteins with a molecular mass of approximately 185-kDa. Further characterisation of the 185-kDa substrate(s) resulted in the cloning of IRS-1 (Sun *et al.*, 1991). IRS-1 contains multiple tyrosine residues in YMXM or YXXM motifs suitable for interaction with *Src* homology 2 (SH2) domain-containing proteins (Shoelson *et al.*, 1992; Sun *et al.*, 1993). IRS-1 also contains over 30 potential serine/threonine phosphorylation sites in motifs recognised by various kinases. A pleckstrin homology (PH) domain is important for association of IRS-1 with the IR (Yenush *et al.*, 1996). IRS-1 and *Shc* also contain a phosphotyrosine binding domain which recognises the NPXY⁹⁶⁰ sequence of the IR (He *et al.*, 1995; Gustafson *et al.*, 1995; Isakoff *et al.*, 1996; Eck *et al.*, 1996). Several specific signalling molecules that interact with tyrosine-phosphorylated IRS-1 have been identified, including PI-3Kinase, phospholipase C γ (PLC γ), growth factor

receptor-bound protein 2 (*Grb2*), SH2-containing phosphotyrosine phosphatases (SHPTP), and *Ras* GTPase (Backer *et al.*, 1993; Skolnik *et al.*, 1993; Lee *et al.*, 1993; Xiao *et al.*, 1994; Yamauchi *et al.*, 1995).

A family of PI-3Kinases phosphorylates the inositol ring at the D-3 position to give PI-3-phosphate from PI, PI-3,4-bisphosphate from PI-4-phosphate, and PI-3,4,5-triphosphate from PI-4,5-bisphosphate (Whitman *et al.*, 1988; Auger *et al.*, 1989). PI-3Kinase is a heterodimer consisting of 85-kDa (α -p85) and 110-kDa (p110) subunits. Cloning of these subunits has revealed that the α -p85 is an adapter subunit containing two SH2 domains and one SH3 domain (Otsu *et al.*, 1991; Skolnik *et al.*, 1991; Escobedo *et al.*, 1991), while the p110 subunit has catalytic activity (Hiles *et al.*, 1992; Hu *et al.*, 1993). It is the presence of the SH2 domains and their demonstrated ability to bind phosphotyrosine that has led to this form of PI-3Kinase being strongly implicated in insulin action in target tissues. It has been shown that IRS-1 associates with the SH2 domain of the 85-kDa regulatory subunit of PI-3Kinase (Myers *et al.*, 1992), and that this association results in the activation of PI-3Kinase because of occupancy of the SH2 domains of p85 by the tyrosine-phosphorylated protein (Myers *et al.*, 1992).

Activation of PI-3Kinase appears to be necessary for the metabolic effects of insulin, including stimulation of glycogen synthesis (Shepherd *et al.*, 1995; Nave *et al.*, 1996; Frevet & Kahn, 1997), glucose transport (Cheatham *et al.*, 1994; Okada *et al.*, 1994; Herbst *et al.*, 1995; Holman & Kasuga, 1997), inhibition of lipolysis (Stagsted *et al.*, 1993; Okada *et al.*, 1994; Moule *et al.*, 1995), stimulation of lipogenesis (Vernon & Lindsay-Watt, 1995), and activation of ACC (Stagsted *et al.*, 1993; Okada *et al.*,

1994; Moule *et al.*, 1995; Travers, *et al.*, 1996) but not pyruvate dehydrogenase by insulin (Kilgour & Vernon, 1987). There is experimental evidence for protein kinase B (PKB) being an element of the signalling cascade leading from PI-3Kinase to activation of glycogen synthesis and glucose transport in isolated rat adipocytes, various cell culture models, and human skeletal muscle (Cross *et al.*, 1995; Hurel *et al.*, 1996; Shepherd *et al.*, 1997). PI-3Kinase activation results in the production of PtdIns(3,4,5)P₃ and PtdIns(3,4)P₂. The binding of the pleckstrin homology domain of PKB to these phosphoinositides both recruits PKB to the plasma membrane and directly and / or indirectly stimulates its kinase activity, *via* phosphorylation by the membrane-associated protein kinases, PDK1/2. Activated PKB phosphorylates substrate proteins resulting in a variety of biological effects, possibly including stimulation of glycogen synthesis, by inhibiting glycogen synthase kinase-3 (GSK3) by serine phosphorylation *via* PKB (Cross *et al.*, 1995), the translation of certain mRNAs (Welsh *et al.*, 1994, 1998) and GLUT4 translocation.

p70^{S6} kinase phosphorylates elongation factor-1 (EF-1) and ribosomal protein S6, and activation of PI-3Kinase is thought to be upstream of p70^{S6} kinase activation and hence mRNA translation (Cheatham *et al.*, 1994), resulting in insulin stimulation of translation elongation (Chang & Traugh, 1997). There is also evidence suggesting that there is PI-3Kinase-independent activation of p70^{S6} kinase by insulin (Hara *et al.*, 1995).

1.5.3 Insulin and signal transduction via MAP kinases

Grb2 is a small cytoplasmic protein that contains two SH3 domains and one SH2 domain that binds to Tyr⁸⁹⁵ in IRS-1. *Grb2* acts as an “adapter molecule” that links

the guanine nucleotide exchange factor for p21^{ras}, termed mSOS (homologous to the *Drosophila* protein, son-of-sevenless (SOS)), to tyrosyl phosphoproteins like the EGF receptor and IRS-1 (Simon *et al.*, 1993; Li *et al.*, 1993). The binding of Grb2/mSOS to IRS-1 mediates the insulin stimulation of p21^{ras}. Ras binds directly to Raf-1 serine/threonine kinase, which in turn activates MAP kinase by phosphorylation and activation of MAP kinase kinase (MEK; Crews & Erikson, 1993).

Shc, another substrate of the IR kinase (Pronk *et al.*, 1993), has three different isoforms of 46-, 52-, and 66-kDa. It has been shown to interact directly with Grb2 after being phosphorylated by the IR (Sasaoka *et al.*, 1994). Therefore, there are at least two pathways by which activation of Ras might occur; that is, IRS-1 and Shc.

MEK causes the activation of the p42 and p44 MAP kinases by threonyl- and tyrosyl-phosphorylation (Ray & Sturgill, 1988). MAP kinases are a conserved family of serine/threonine kinases that are activated in all eukaryotic cells in response to a wide range of signals (Lenormand *et al.*, 1993; Marshall, 1995). MAP kinases then phosphorylate and activate p90rsk (Haystead *et al.*, 1990). MAP kinase itself translocates into the nucleus upon activation by a number of mitogens, where it is thought to phosphorylate transcription factors such as p62^{TCF}, leading to stimulation of activating protein-1 (AP-1) activity. However, it is likely that a large fraction of the activated enzyme remains cytoplasmic, and contributes to some metabolic processes. MAP kinase catalyses the rephosphorylation of upstream proteins in the pathway, such as Sos, causing the desensitisation of p21^{ras} activity (Waters *et al.*, 1995).

A central role for MAP kinase in growth factor and insulin signalling is probable because MAP kinase can phosphorylate a large number of target substrates *in vitro*,

including p90 S6 kinase (Sturgill *et al.*, 1988), MAPKAP kinase-2 (a kinase that phosphorylates glycogen synthase at Ser7 ; Stokoe *et al.*, 1992), EGF receptor (Takishima *et al.*, 1991), PLA₂ (Lin *et al.*, 1993), tyrosine hydroxylase (Sutherland *et al.*, 1993a), PHAS-1 (Lin *et al.*, 1994), protein tyrosine phosphatase 2C (Peraldi *et al.*, 1994), stathmin (Leighton *et al.*, 1993) and the transcription factors *c-myc*, *c-jun*, *c-fos*, p62^{TCF}, NF-IL6 and ATF-2 (Nishida & Gotoh). The role of MAP kinase in the metabolic effect of insulin, however, is uncertain (Denton & Tavaré, 1995).

1.6 Phosphodiesterases (PDEs)

In adipocytes, the intracellular concentrations of cyclic AMP are controlled by the complex coordinate regulation of both adenylate cyclase and cyclic AMP PDE activities. Changes in intracellular cyclic AMP greatly affect the metabolic status of the cell. Enzyme activity that catalyses the hydrolysis of cyclic AMP and cyclic GMP was described almost immediately after the discovery that cyclic nucleotides exist in the cell. In fact, the demonstration of enzymatic degradation of cyclic nucleotides by PDEs formed part of the criteria that these compounds act as second messengers. Within a few years it became apparent that more than one enzyme was able to catalyse the hydrolysis of cyclic AMP and cyclic GMP. We now know that many different PDEs selectively catalyse the hydrolysis of purine 3',5' nucleoside monophosphates to 5'-nucleoside monophosphates. By catalysing the degradation of cyclic AMP and cyclic GMP, cyclic nucleotide PDEs are critical determinants in regulating intracellular concentrations and, consequently, biological effects of these important second messengers. PDEs are now thought of as mediators of "cross-talk" between different second messenger signalling systems (see Beavo & Houslay, 1990).

PROPERTIES OF PDE FAMILIES

FAMILY	(specific inhibitor)	K_m	cDNA/isozyme name	intracellular regulatory properties
1	Ca ²⁺ /calmodulin-sensitive (Methoxymethyl-IBMX)	cGMP<=cAMP	PDE1A/1A1, 1A2 PDE1B/1B1 PDE1C/1C1, 1C2, 1C3, 1C4, 1C5	Ca ²⁺ , calmodulin Phosphorylation by cAMP-kinase (PKA), CaM-kinase
2	cGMP-stimulated Positive cooperativity Non-catalytic binding sites	cGMP<=cAMP	PDE2A/2A1, 2A2	cGMP
3	cGMP-inhibited (Ionotropic/vasodilator drugs, e.g., cilostamide, milrinone, Enoximone, Imazodan, LY195115 (Indolidan))	cAMP<=cGMP	PDE3A/3A1 PDE3B/3B1	cGMP Phosphorylation by cAMP-kinase (PKA) or insulin-activated kinases
4	cAMP-specific (Rolipram, RO 20-1724)	cAMP<<=cGMP	PDE4A/4A1, 4A5, 4A8 PDE4B/4B1, 4B2 PDE4C/4C1 PDE4D/4D1, 4D2, 4D3, 4D4	cAMP (gene expression) Phosphorylation by cAMP-kinase (PKA)
5	cGMP-specific (Dipyridamole, Zaprinast)	cGMP<<<=cAMP	PDE5A/5A1	
6	cGMP-specific Photoreceptor cGMP-binding	cGMP<<<=cAMP	PDE6A/6A1 PDE6B/6B1	Transducin (G-protein) Phosphorylation by cGMP-kinase
7	Non-catalytic binding sites cAMP-specific rolipram-insensitive	cAMP<<=cGMP	PDE7A/7A1, 7A2	
8	cAMP-specific IBMX-insensitive (Dipyridamole)	cAMP<<=cGMP	PDE8	cAMP
9	cGMP-specific	cGMP<<<=cAMP	PDE9A1	
10	cAMP/cGMP	cAMP<<=cGMP	PDE10A	

Table 1.1. Properties of PDE families (adapted from Manganiello *et al.* (1995) *Cell.Signal.*, **7**. 445-455)

In individual cells, cyclic nucleotide hydrolysis is a highly regulated process, usually dependent on the coordinated and integrated activities of several structurally related PDE isoenzymes. Cyclic nucleotide PDEs are a multienzyme family in which at least 14 distinct genes encode over 20 different isoforms. These isoforms have been classified into ten families according to their substrate specificity, sequence similarities and regulatory sites (see table 1.1) (Thompson, 1991; Torphy *et al.*, 1993; Bolger, 1994; Manganiello *et al.*, 1995; Beavo, 1995; Conti *et al.*, 1995; Dent & Giembycz, 1995; Houslay & Milligan, 1997; Souness & Rao, 1997; Fisher *et al.*, 1998; Soderling *et al.*, 1998a,b; Soderling *et al.*, 1999). Although, sometimes, specific PDEs are highly concentrated in single types of cells, multiple PDEs are usually present in most cells, in different amounts, proportions and subcellular locations. Some PDE forms are specific for cAMP, such as the PDE3, PDE4 and PDE7 families. Members of the PDE1 and PDE2 families can hydrolyse both cAMP and cGMP. This chapter will focus on what are currently believed to be the major and key cAMP-hydrolysing PDEs of adipocytes, namely PDE3 and PDE4.

1.6.1 Structure and function of PDEs

Members of PDE families are products of distinct, but related, genes. PDEs share a common structural domain pattern, all containing conserved catalytic domains (~25-40% amino acid identity) of ~270 amino acids, usually in the C-terminal portion of the enzymes (Charbonneau *et al.*, 1986; Charbonneau, 1990). The seven known families of PDEs that can hydrolyse cAMP (PDE-1,-2,-3,-4,-7,-8 and -10) have a central core region of ~330 amino acids containing the active site. The N-terminal regulatory domains are divergent which accounts for the distinctive regulatory properties unique to the individual families, i.e. putative Ca^{2+} -calmodulin binding

sites, membrane-associated domains, allosteric non-catalytic cGMP-binding sites, putative phosphorylation sites for Ca^{2+} -, insulin-, cAMP- and cGMP-dependent protein kinases (Charbonneau *et al.*, 1986; Beavo, 1988; Beavo & Houslay, 1990; Beavo & Reifsnyder, 1990; Charbonneau, 1990; Conti *et al.*, 1991; Bentley & Beavo, 1992; Nicholson *et al.*, 1991; Thompson, 1991; Torphy & Udem, 1991; Giembycz, 1992; Hall, 1993).

1.6.2 PDEs and adipocytes

Although sometimes specific PDEs are highly concentrated in single types of cells, multiple PDEs are usually present in most cells, in different amounts, proportions and subcellular locations. The PDE3 family has two different gene products that have currently been identified (Meacci *et al.*, 1992; Taira *et al.*, 1993). PDE3A has been identified in smooth muscle, platelets (MacPhee *et al.*, 1986; Alvarez *et al.*, 1986; LeBon *et al.*, 1992; Degerman *et al.*, 1995) and cardiac tissue. PDE3B is mainly located in adipocytes (Kono *et al.*, 1975; Degerman *et al.*, 1987; Anderson *et al.*, 1989) and liver (Pyne *et al.*, 1987; Boyes & Loten, 1988), although both PDE3A and PDE3B are found in lower amount in other tissues. In liver (Pyne *et al.*, 1987; Boyes & Loten, 1988) and adipocytes (Kono *et al.*, 1975; Degerman *et al.*, 1987; Anderson *et al.*, 1989), PDE3B is predominantly membrane-associated. PDE3 has high affinity for both cAMP and cGMP, and low concentrations of cGMP can inhibit the hydrolysis of cAMP by this enzyme.

The PDE4 isoforms are widely expressed in many tissues. They have a high affinity for cAMP, but practically no activity with cGMP as substrate. However, unlike PDE3, cGMP does not bind to PDE4 with high affinity. PDE4 is the largest PDE

family, with 4 different genes having been identified in human and rat. All of these genes are reported to encode more than one alternatively spliced mRNA transcript. It has been shown that increased PDE4 activity in the mouse can be responsible for one form of diabetes insipidus (Homma *et al.*, 1991).

It has recently been shown by MacKenzie and colleagues that the cAMP-specific PDE4A5 can be activated by stimulation of p70 S6 kinase *via* a GH-controlled PI-3Kinase pathway in 3T3 pre-adipocytes (MacKenzie *et al.*, 1998). This identified a role for the PDE4 phosphodiesterases in regulating the ability of GH to stimulate differentiation of 3T3-F442A cells (MacKenzie *et al.*, 1998). However, PDE3 isoform inhibitors did not enhance differentiation to adipocytes (MacKenzie *et al.*, 1998). Elks and Manganiello have also shown that PDE4, and not PDE3, inhibitors enhance differentiation to adipocytes (Elks & Manganiello, 1985) in 3T3-L1 fibroblasts. They also showed that PDE3, but not PDE4, inhibitors played an important role in regulating lipolysis in the mature adipocytes (Elks & Manganiello, 1985). This suggests that the differences in response to these selective PDE inhibitors reflect compartmentalisation of cAMP signalling between a pool controlled by PDE3 activity and another pool controlled by PDE4 activity. Studies on other cell types also support this notion (Faux & Scott, 1996; Erdogan & Houslay, 1997; Houslay & Milligan, 1997).

1.6.3 PDEs and lipid metabolism

Insulin and catecholamines play a major role in the acute regulation of adipose tissue lipolysis (Arner, 1988). One important effect of the insulin signal is to mediate activation of membrane-associated cGMP-inhibited PDE (PDE3). This leads to a

reduction in cAMP, which results in a decrease in cAMP-dependent protein kinase (PKA) and a reduction in phosphorylation, which attenuates HSL activity, the rate-limiting enzyme in the regulation of lipolysis (Beebe *et al.*, 1985; Londos *et al.*, 1985; Elks & Manganiello, 1985; Nilsson *et al.*, 1986; Schmitz-Peiffer *et al.*, 1992; Eriksson *et al.*, 1995; Manganiello *et al.*, 1995). This reduction in HSL activity can occur by the downregulation of β -adrenoceptors (Engfeldt *et al.*, 1988) or activation of cAMP phosphodiesterase (PDE) (Manganiello & Vaughan, 1973).

During recent years, a number of studies have supported the idea that insulin-induced phosphorylation and activation of PDE3 is critical to the antilipolytic and antiglycogenolytic effects of insulin in adipose tissue (Manganiello *et al.*, 1991; Manganiello *et al.*, 1992), although this has mainly been based on *in vitro* findings. Hägström-Toft and colleagues presented a report on the effects on lipolysis in human adipose tissue *in vivo* of nonselective and selective (PDE3) inhibition of PDE (Arner *et al.*, 1993) found using a microdialysis method, showing that PDE3 inhibition with a selective inhibitor stimulates lipolysis, but not to the same extent as a nonselective PDE inhibitor. This suggests that other PDE isoforms are involved in the regulation of lipolysis. The antilipolytic effects of insulin have been shown to be abolished by using nonselective PDE inhibitors (Fain & Rosenberg, 1972; Makino *et al.*, 1992). This was also observed using a selective inhibitor of PDE3 (Smith *et al.*, 1991; Manganiello *et al.*, 1991; Manganiello *et al.*, 1992), whereas the inhibition of PDE4 did not inhibit antilipolysis by insulin (Schechter, 1984; Gabbay & Lardy, 1986). In liver, insulin has been reported to cause the activation of both the PDE3 "dense vesicle" and also a PDE4 isoform (Heyworth *et al.*, 1983). This indicated that

multiple PDEs may be involved in the ability of insulin to regulate cAMP signalling processes (Houslay & Kilgour, 1990).

The mechanism whereby insulin activates PDE3 is still not fully understood. However, rapid regulatory changes in cAMP can be elicited by the phosphorylated PDE3 enzymes (Manganiello *et al.*, 1982; Kilgour *et al.*, 1989; Houslay & Kilgour, 1990; Degerman *et al.*, 1990; Manganiello *et al.*, 1991; Smith *et al.*, 1991; Manganiello *et al.*, 1995) and isoforms of PDE4 enzymes (Marchmont & Houslay, 1980; Sette *et al.*, 1994). Early studies with PDE3 indicated that both an increase in cyclic AMP by isoproterenol and insulin resulted in the phosphorylation/activation of PDE3, and that the effect was more than additive (Smith & Manganiello, 1988; Smith *et al.*, 1991) suggesting cross-talk between the two signal transduction pathways. An insulin-sensitive PDE3 serine kinase was then identified (Shibata & Kono, 1990a; Shibata & Kono, 1990b; Smith *et al.*, 1992; Lopez-Aparacio *et al.*, 1993). This kinase was not a substrate for direct phosphorylation by the activated insulin receptor (Lopez-Aparacio *et al.*, 1993), but possibly belonged to a family of protein serine kinases regulated by upstream kinases/phosphatases involved in insulin receptor-mediated signal transduction cascades. Based on the deduced amino acid sequence of adipocyte PDE3 (Taira *et al.*, 1993), Rascon *et al.* (1994) found that a probable site for phosphorylation of PDE3 by PKA was Ser⁴²⁷, but this was in a cell-free system. Rahn *et al.* (1996) then showed that a single site in PDE3 (Ser³⁰²) was phosphorylated in adipocytes incubated with isoproterenol and/or insulin. There was some confusion as to whether PKA and insulin resulted in phosphorylation of different sites or the same site on PDE3. The reason for the discrepancy is probably due to adipocyte PDE3 being membrane-bound. It is possible that ser⁴²⁷ is usually inserted in the membrane,

and therefore not subject to phosphorylation in intact cells. In the cell-free system (Rascon *et al.*, 1994), ser⁴²⁷ is exposed, and can therefore be phosphorylated.

Data was then published showing effects of pretreatment of adipocytes with the PI-3kinase inhibitor, wortmannin. Wortmannin is known to inhibit the antilipolytic action of insulin (Okada *et al.*, 1994), as well as other effects of insulin and other growth factors, like regulation of cell growth (Myers & White, 1993; Parker & Waterfield, 1993; White & Kahn, 1994; Van Horn *et al.*, 1994; Keller & Lienhard, 1994). Pre-treatment of adipocytes with wortmannin inhibited phosphorylation/activation of PDE3B, blocked insulin-induced activation of a kinase that phosphorylates PDE3B (Rahn *et al.*, 1994) and the antilipolytic action of insulin (Okada *et al.*, 1994; Rahn *et al.*, 1994). However, the components in the signalling pathway between PI-3Kinase and PDE3B, including the kinase responsible for the phosphorylation of PDE3B, had not been identified. Wijkander and colleagues (Wijkander *et al.*, 1998) have now shown that PKB is responsible for the phosphorylation of PDE3B *in vitro*. This has since been found by others (Kitamura *et al.*, 1999), and is consistent with one site on PDE3 being phosphorylated by insulin and PKA (Serine 302).

Longer-term changes in cAMP levels, through up-regulation, have also been described as adaptive responses to chronic increases in intracellular cAMP levels (Thompson, 1991; Conti *et al.*, 1995a). This has been described in particular detail for splice variants arising from the PDE4D gene (Sette *et al.*, 1994; Conti *et al.*, 1995a; Conti *et al.*, 1995b), where short PDE4D1 splice variants are upregulated, suggesting distinct promoter regions.

The diversity among PDE isoforms may provide unique mechanisms for regulating the amplitude and duration of cyclic nucleotide levels in particular cells. Targeting of specific cell types by selective inhibitors for different splice variants may provide a pharmacological approach to the treatment of a wide variety of metabolic disorders.

1.7 Objectives

The aim of this study was to further elucidate the mechanisms whereby GH exerts its chronic effect on adipose tissue metabolism, in particular the effect of GH on lipolysis.

A study by Green *et al.* (1994) had shown that $\text{TNF}\alpha$ chronically increases basal lipolysis in rat epididymal adipocytes: an effect that is similar to that of GH. Borland *et al.* (1994) had implicated a protein with a half-life of less than 3 hours in the regulation of lipolysis and lipogenesis by GH. This raised the possibility that $\text{TNF}\alpha$ might mediate the chronic metabolic effects of GH on adipose tissue, an analogous system to the mediation of the mitogenic effects of GH by IGF-1. Initial studies using ovine adipose tissue explants involved the investigation if $\text{TNF}\alpha$ could be this putative secreted protein. However, $\text{TNF}\alpha$ did not appear to mimic the effects of GH on isoproterenol-stimulated lipolysis in this system, and therefore I concluded that $\text{TNF}\alpha$ was not the protein involved in GH regulation of lipolysis and lipogenesis.

Previous studies in the laboratory on the mechanism of GH action had used inhibition of signal transduction components in an explant system. More specific effects could be observed by using an antisense approach, but this required the use of a cell culture

system rather than adipose tissue explants. The next objective of the study was then to determine the suitability of an ovine adipose cell culture system for investigating the molecular basis of the lipolytic effects of GH; in particular the inhibitory effects of GH on adenosine inhibition of lipolysis. The lipolytic system partially developed in primary ovine adipocytes, but only a very small response to adenosine was observed under sub-optimum conditions. This response to adenosine was considered too small to investigate the effects of GH. However, by manipulating the differentiation conditions, I significantly improved both cell differentiation and the lipolytic response and sensitivity to isoproterenol, but there was no improvement in response to adenosine.

As ovine primary adipocytes only had a small response to adenosine, the suitability of the murine cell line, 3T3-F442A, was investigated for determining the molecular basis of the lipolytic effects of GH. However, although the lipolytic system did develop in differentiated 3T3-F442A adipocytes and response to isoproterenol was observed, no significant effect of adenosine was measured in this system either. This line of investigation was not pursued further.

Therefore, I decided to investigate the effects of GH and insulin on the lipogenic system in 3T3-F442A adipocytes instead. This was with a view to extending previous observations by others (Millar, 1998) in the laboratory on the roles of specific isoforms of PKC on the modulation of lipogenesis by insulin and GH. The main objective was to determine the role of PKC isoforms in the modulation of the effect of insulin and GH on the lipogenic enzyme, ACC, and its mRNA, total ACC, proportion of active ACC. However, the effect of the hormones on lipogenesis, and especially

ACC, was considered to be too small to investigate the roles of specific PKC isoforms, despite trying many different ways of improving the hormone effects.

A possible explanation for the poor response to insulin could be that the lipogenic system was not being "switched off" in the absence of stimulant, so isoproterenol was added to the 3T3-F442A adipocytes to decrease lipogenesis. Isoproterenol did reduce the rate of lipogenesis, but the effect of insulin was still small. Therefore, modulation of the effect of specific PDE isoforms on lipogenesis was explored as an alternative.

CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals and reagents

General laboratory chemicals were purchased from Sigma Chemical Company (Poole, Dorset, UK), Gibco BRL (Renfrewshire, Scotland), BDH (Poole, Dorset, UK) or Calbiochem (Nottingham, UK), unless otherwise stated.

Tissue culture plastic was obtained from Corning Costar Corporation (Buckinghamshire, UK) or Greiner Labortechnik Ltd. (Dursley, UK). Tissue culture media, antibiotics, collagenase (type II) and serum were obtained from Life Technologies (Paisley, Scotland), Sigma Chemical Company (Poole, Dorset, UK) or Labtech International (Uckfield, East Sussex, UK). Pentex Ex-cyte III was purchased from Bayer Corporation (Basingstoke, Hampshire, UK).

Bovine growth hormone was a generous gift given by Monsanto Europe, B-1150 (Brussels, Belgium).

[^{14}C]-acetate and [$\text{U-}^{14}\text{C}$]-glucose were obtained from ICN Pharmaceuticals, Inc. (Hertfordshire, UK) and sodium[^{14}C]bicarbonate was purchased from Amersham International plc (Buckinghamshire, UK). Scintillation cocktails and vials were purchased from Packard (Berkshire, UK) or ICN Pharmaceuticals, Inc (Herts, UK).

2.1.2 Preparation of bovine serum albumin

The bovine serum albumin (BSA) used for all the tissue or cell culture and relevant experiments was fraction V, essentially fatty acid-free. This was dialysed prior to use to remove contaminants such as citrate and pyruvate, as described by Hanson &

Ballard (1968). A 10% BSA solution made up in 0.9% NaCl solution was dialysed against 0.9% NaCl at 4°C for a period of 3 days. The 0.9% NaCl solution was changed 5 times over this period, with a minimum of 4 hours between each change. The BSA was finally dialysed for a minimum of 4 hours against distilled water before being lyophilised, and the dry powder was stored at 4°C until required.

2.1.3 Cell lines

3T3-F442A cells were generously provided by Dr. Howard Green (Harvard Medical School, USA)

2.1.4 Sheep

Sheep were all Finn x Dorset cross-breds. Wether (castrated male) lambs of 1 month old to 9 months old were used. The animals were given hay, with water freely available, and a cereal mix (the amount depending on age); young lambs were left to feed naturally with their mothers, for at least 4 weeks before slaughter. Lactating sheep were killed after 18 days of lactation, and were fed on hay and a cereal mix, as described previously, prior to slaughter (Vernon *et al.*, 1981). Sheep were anaesthetised with an intrajugular injection of 10-30 ml of Sagatal (May & Baker, Dagenham, Essex, UK) depending on age, administered by Dr. R.G. Vernon. The animals were exsanguinated when unconscious, and samples of adipose tissue were removed. These were then placed in a sterile isotonic saline at 37°C and taken to the laboratory immediately.

2.1.5 Rats

Male Wistar rats (A. Tuck and Son, Rayleigh, Essex, UK) were fed on standard pelleted rat chow *ad libitum* (CRM diet, Labsure, Poole, UK), with water freely available. Rats of similar age and weight (150-180g) were used for experimental purposes. Rats were anaesthetised by an intraperitoneal injection of 0.9ml Sagatal plus 0.1ml Hypnorm (May & Baker, Dagenham, Essex, UK), administered by Dr. R.G. Vernon. The animals were exsanguinated when unconscious, and samples of adipose tissue were removed. These were then placed in isotonic saline at 37°C and taken to the laboratory immediately.

2.2 Methods

2.2.1 Tissue culture

2.2.1.1 Preparation of ovine explant tissue

Pieces of either subcutaneous or popliteal ovine adipose tissue (approximately 20mg) were cut with sterilised scissors, after the removal of blood vessels and connective tissue. Tissue explants giving a total weight of around 100mg were maintained in culture at 37°C for 22 hours in an atmosphere of 95% air, 5% CO₂. The tissue explants were cultured in 24 well plates in 2mls of Medium 199 containing Earle's salts, L-glutamine and 25mM Hepes (pH 7.3) and supplemented with 2mM acetate (pH 7.3) and antibiotics (Penicillin G, 60µg/ml ; streptomycin sulphate, 100µg/ml ; neomycin sulphate, 10µg/ml) (Vernon & Finley, 1988). The tissue was then transferred to fresh medium for a further 24 hour period, containing the relevant additions for the experiments, as described in the results chapters.

2.2.1.2 Preparation of rat explant tissue

Pieces of rat epididymal adipose tissue (approximately 15mg) were cut with sterilised scissors, after the removal of blood vessels and connective tissue. Tissue explants giving a total weight of around 100mg were maintained in culture at 37°C for 22 hours in an atmosphere of 95% air, 5% CO₂. The tissue explants were cultured in 24 well plates in 2mls of Medium 199 containing Earle's salts, L-glutamine and 25mM Hepes (pH 7.3) and supplemented with 2mM acetate (pH 7.3) and antibiotics, as with the sheep explant culture. The tissue was then transferred to fresh medium for a further 24 hour period, containing the relevant additions for the experiments, as described in the results chapters.

2.2.2 Cell culture

2.2.2.1 Preparation of ovine pre-adipocytes

Pieces of subcutaneous ovine adipose tissue (approximately 20mg) were cut with sterilised scissors, after the removal of blood vessels and connective tissue, in Kreb's Ringer's bicarbonate medium (1.2mM CaCl₂, 120mM NaCl, 5mM KCl, 1.5mM KH₂PO₄ and 25mM NaHCO₃) supplemented with 1mg/ml D-glucose, 2mM acetate, 25mM Hepes (pH 7.4), and 3% BSA (the Ringer's bicarbonate medium was gassed for 20 minutes with 5% CO₂, 95% O₂). The explants were subjected to digestion with collagenase. The collagenase digest was made up in Ringer's bicarbonate medium supplemented with 1mg/ml D-glucose, 2mM acetate, 25mM Hepes, 4% BSA and 1mg/ml collagenase, and filtered through a 0.45µm filter into a conical tissue culture flask. The tissue explants were washed in Ringer's bicarbonate medium and transferred to the flask containing the collagenase digest, before being placed in a shaking water bath at 37°C for 1 hour. After the digestion, the digest was filtered into

sterile 50ml tubes using coarse mesh and a siliconised funnel, and the adipocytes were allowed to float to the upper layer of the digest. The infranatant was removed to fresh tubes, and centrifuged at room temperature for 5 minutes at 2800 rpm in an MSE benchtop centrifuge, to give a pellet of stromovascular cells and red blood cells. The supernatant was removed, and the pellet was resuspended in 30mls of warm Hank's Balanced Salt Solution without Calcium or Magnesium (HBSS). The cells were centrifuged and resuspended twice more as previously, but the cells were only in 20mls of HBSS the last time. The cell suspension was filtered through a fine mesh, and 40 μ l of the filtered cell suspension was removed to count cell number using a haemocytometer. The cell suspension was centrifuged for 5 minutes at 2800 rpm, and the pellet resuspended in the appropriate amount of proliferation medium to give a cell number of 3×10^5 cells/ml. The proliferation medium consisted of Medium 199 containing Earle's salts, L-glutamine and 25mM Hepes (pH 7.3) and supplemented with 2mM acetate (pH 7.3), antibiotics (Penicillin G, 60 μ g/ml ; streptomycin sulphate, 100 μ g/ml) and 20% Newborn Calf Serum (NCS). 16mls of proliferation medium and 4mls of cell suspension were added to a 75cm² tissue culture flask to give a final cell concentration of 0.6×10^5 cells/ml.

2.2.2.2 Culture of ovine pre-adipocytes

24 hours after the preparation of the pre-adipocyte cells, the cells were washed with HBSS and the medium was replaced with fresh proliferation medium. This was repeated every 48 hours thereafter until the cells had reached 80% confluence (determined visually) before they entered growth arrest. If required, the cells were passaged using 1ml of 0.25% trypsin / EDTA solution, and were transferred to 24 well

plates at a density of 0.6×10^5 cells/ml in 2mls proliferation medium. The medium was replaced with fresh proliferation medium every 48 hours.

2.2.2.3 Differentiation of ovine pre-adipocytes

For conversion to adipocytes, the ovine pre-adipocytes were grown to confluence in the 24 well plates. Two day confluent cultures were induced to differentiate by replacing the proliferation medium with differentiation medium. This medium consisted of Dulbecco's Modified Eagle's Medium (DMEM) / Ham's F12 supplemented with penicillin ($60 \mu\text{g/ml}$), streptomycin ($100 \mu\text{g/ml}$) and typically Ex-cyte III (1% v/v), Insulin (10ng/ml) and T_3 (2nM), unless otherwise stated. The medium was only replaced with fresh medium every 4 days.

2.2.2.4 Culture of 3T3-F442A pre-adipocytes

3T3-F442A pre-adipocytes were cultured in Dulbecco's Modified Eagle's Medium (DMEM) containing 2mM glutamine and supplemented with 100units/ml penicillin, $100 \mu\text{g/ml}$ streptomycin (pen/strep) and 10% (v/v) Newborn Calf Serum (NCS) (growth medium). The cell were grown in a 90% O_2 :10% CO_2 atmosphere at 37°C . Stock cultures were passaged routinely at approximately 80% confluence using 1ml of 0.25% trypsin / EDTA solution. For experimental purposes, pre-adipocytes were seeded at 0.1×10^6 cells, and grown to confluence in 24 well plates, unless stated otherwise. The growth medium was changed every 48 hours.

2.2.2.5 Differentiation of 3T3-F442A pre-adipocytes

To convert the 3T3-F442A pre-adipocytes to adipocytes, the pre-adipocytes were used between passages 6 and 14, and grown to confluence. Two day confluent cultures

were induced to differentiate by replacing the growth medium with DMEM containing 2mM glutamine, supplemented with pen/strep, 10% Foetal Calf Serum (FCS) and 5 μ M insulin (28.7 μ g/ml) (differentiation medium). The differentiation medium was replaced with fresh differentiation medium every 72 hours. Under these conditions, at least 80% of the pre-adipocytes converted to adipocytes by day 8.

2.2.3 Procedures using ovine and rat explant tissue

2.2.3.1 Lipolysis assay

Ovine or rat adipose tissue explants were prepared (as described earlier), and the rate of lipolysis of either fresh tissue or after tissue culture was measured as glycerol release during a 3 hour incubation period at 37°C in 2.5mls Ringer's bicarbonate medium supplemented with 1mg/ml D-glucose, 2mM acetate, 25mM Hepes (pH 7.4) and 3% BSA (dialysed and fatty acid free) as described by Vernon & Finley (1985). The incubation medium was supplemented with hormones and other agents, as described in the results chapters. After the 3 hour incubation period, 1ml of the medium was removed and transferred to NA5 tubes containing 120 μ l of 45% (w/v) perchloric acid (on ice) to deproteinise it. This was followed by a 15 minute centrifugation period at 2500 rpm in an MSE benchtop centrifuge. 800 μ l of the supernatant was removed and transferred to LP4 tubes containing 85 μ l 5M KOH and 170 μ l of saturated KHCO₃. These were then mixed and centrifuged again for 15 minutes at 2500 rpm. Following this step, 800 μ l of the supernatant was removed to fresh LP3 tubes and stored at -20°C prior to the determination of glycerol content. The weight of the tissue explants from each sample was recorded by blotting them and weighing them prior to them being discarded.

2.2.3.2 Glycerol assay

Glycerol concentration was measured as described previously (Aitchison *et al.*, 1982). The samples were defrosted and centrifuged for 15 minutes at 2500 rpm in an MSE benchtop centrifuge. After this period, 30 μ l of each supernatant was placed in triplicate into a 96 well plate, and 200 μ l of assay mix was added to each well. This consisted of 13.5ml of 0.25M triethanol ammonium chloride buffer (pH 7.5), 2.49ml of 2mg/ml NAD, 2.71ml of 5mg/ml ATP, 248 μ l of 1M MgCl₂, 12 μ l of Triton X-100 (10%), 212 μ l of 1mg/ml Diaphorase, 20 μ l of 10mg/ml Glycerol-3-phosphate dehydrogenase (G-3-PDH), 808 μ l of distilled water and 8mg of [3-{4,5dimethylthiosal-2-yl}-2,5-diphenyl-tetrazoliumbromide] (MTT). The assay mix was made up in a light-protected bottle containing a magnesium flea, and was stirred at 4⁰C. Glycerol levels were determined by the addition of 50 μ l of glycerol kinase (4units/ml) to each well, and the plates were incubated at room temperature for 45 minutes in the dark. After this period, the plate was read at 600nm absorbance on a Titertek plate reader. Glycerol levels were calculated from a glycerol standard curve that was measured on each 96 well plate over a concentration range of 2nm to 20nm. For tissue explants, the number of adipocytes per gram of tissue was determined, as described in section 2.2.6. The rate of glycerol release was expressed as μ mol glycerol / hr / 10⁶cells.

2.2.3.3 Lipogenesis assay

Adipose tissue explants were prepared (as described previously) and various hormones and / or other agents were added to the culture medium, as indicated in the results chapters. The rate of lipogenesis of fresh tissue and tissue after culture was

determined by measuring the amount of ^{14}C -labelled sodium acetate incorporated into the cell lipid over a 2 hour period, using the method described by Vernon & Finley (1988). Prior to the measurement of the rate of lipogenesis, the tissue was transferred to 24 well plates containing 2.5ml of fresh culture medium. 100 μl of 2.5 $\mu\text{Ci/ml}$ [^{14}C]-acetate was added to each of the wells for a 2 hour period. The tissue was then removed from the medium, blotted on filter paper, and weighed. To obtain blank values, 100 μl of 2.5 $\mu\text{Ci/ml}$ [^{14}C]-acetate was added to 3 wells containing tissue explants for one minute before the tissue was blotted and the dry weight taken. The tissue was then transferred to a glass mini-scintillation vial, and the samples were dissolved in a scintillation cocktail comprising of 2ml of toluene plus 4ml of opti-fluor scintillants. The samples were vortexed for 10 seconds each, and counted in a Packard 1600 TR liquid scintillation analyser. To obtain a value for total counts, 10 μl of medium was removed from 4 wells at random and transferred to glass mini-scintillation vials. 4ml of emulsifier safe was added and the total counts were counted in a Packard 1600 TR liquid scintillation analyser. The number of adipocytes per gram of tissue was determined, as described in section 2.2.6. The rate of lipogenesis was expressed as nmol acetate incorporated / hr / 10^6 cells.

2.2.4 Procedure using ovine adipocyte cells

2.2.4.1 Lipolysis assay

Differentiation medium was removed from the wells, the cells were washed once with 0.5ml of PBS, and the medium was replaced with 1ml Ringer's bicarbonate medium supplemented with 1mg/ml D-glucose, 2mM acetate, 25mM Hepes (pH 7.4) and 3% BSA (dialysed and fatty acid free). The rate of lipolysis was measured as glycerol release during a 3 hour incubation period at 37°C in a modification of the method

described by Vernon & Finley (1985). After the 3 hour incubation period, 800µl of the medium was removed and transferred to NA5 tubes containing 96µl of 45% (w/v) perchloric acid (on ice) to deproteinise it. This was followed by a 15 minute centrifugation period at 2500 rpm in an MSE benchtop centrifuge. 600µl of the supernatant was removed and transferred to LP4 tubes containing 64µl 5M KOH and 128µl of saturated KHCO₃. These were then mixed and centrifuged again for 15 minutes at 2500 rpm. Following this step, 600µl of the supernatant was removed to fresh LP3 tubes and stored at -20°C prior to the determination of glycerol content (section 2.2.3.2).

2.2.4.2 Determination of cell number

After the medium had been removed for the lipolysis assay, the cell number was determined for each well by detaching the cells using 0.25% trypsin / EDTA solution for 1 minute, resuspending the cells in 20ml of proliferation medium, and counting the cell number using a haemocytometer.

2.2.5 Procedure using 3T3-F442A adipocyte cells

2.2.5.1 Lipolysis assay

Differentiation medium was removed from the cells and replaced with differentiation medium minus FCS and insulin 8 days after the induction of adipogenesis, unless stated otherwise. After an overnight period, growth hormone (100ng/ml) and / or insulin (100ng/ml) were added to the medium for either 24 hours or 48 hours, as indicated in the results chapters. Prior to the measurement of the rate of lipolysis, the medium was removed, the cells washed once with 0.5ml of PBS, and the rate of

lipolysis was measured as glycerol release during a 3 hour incubation period at 37°C in 1ml Ringer's bicarbonate medium supplemented with 1mg/ml D-glucose, 2mM acetate, 25mM Hepes (pH 7.4) and 3% BSA (dialysed and fatty acid free). The amount of glycerol released was determined as in section 2.2.3.2.

2.2.5.2 Lipogenesis assay [¹⁴C]acetate

Differentiation medium was removed from the cells and replaced with differentiation medium minus FCS and insulin 8 days after the induction of adipogenesis, unless stated otherwise. After an overnight period, growth hormone (100ng/ml) and / or insulin (100ng/ml) were added to the medium for either 24 hours or 48 hours, as indicated in the results chapters. Prior to the measurement of the rate of lipogenesis, the medium was removed, the cells washed once with 0.5ml of PBS, and the medium replaced with fresh 1ml of fresh DMEM (4.5g/l glucose, unless otherwise stated), containing 2mM glutamine and supplemented with 100 units/ml penicillin and 100µg/ml streptomycin (pen/strep). The rate of lipogenesis was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period (unless indicated otherwise), using a modification of the method described by Vernon & Finley (1988). 1µCi/ml [¹⁴C]-acetate + 2mM cold sodium acetate were added to the medium for a 4 hour period, after which time the medium was removed, the cells were washed once before being lysed by the addition of GIT buffer (0.1M EDTA pH 8.0, containing 5M guanidine thiocyanate, Melford Laboratories Ltd., Suffolk, UK). To obtain blank values, 100µl of 1µCi/ml [¹⁴C]-acetate + 2mM cold acetate were added to 3 wells containing cells for one minute before the medium was removed and the cells were lysed. Lysates were transferred to 1.5ml eppendorf tubes, and lipids were extracted by the addition of an equal volume

of water-saturated chloroform. The samples were mixed well and centrifuged at 10,000g for 5 minutes in an eppendorf centrifuge (5402). The upper aqueous layer was carefully removed and kept separate for the determination of DNA content. The lipid-containing chloroform layer was transferred to a glass mini-scintillation vial and allowed to dry overnight. Samples were then dissolved in a scintillation cocktail comprising of 1ml of toluene scintillation fluid and 4ml of EcoLite (ICN). To obtain a value for total counts, 10 μ l of medium was removed from 4 wells at random and transferred to glass mini-scintillation vials. 4ml of emulsifier safe was added. The samples were counted in a Packard 1600 TR liquid scintillation analyser, and the rate of lipogenesis was expressed as μ mol acetate incorporated / hr / mg of DNA. The amount of DNA was determined, as described in section 2.2.5.4.

2.2.5.3 Lipogenesis assay [14 C]glucose

Differentiation medium was typically removed from the cells and replaced with differentiation medium minus FCS and insulin 8 days after the induction of adipogenesis, unless stated otherwise. After an overnight period, growth hormone (100ng/ml) and / or insulin (100ng/ml) were added to the medium for either 24 hours or 48 hours, as indicated in the results chapters. Prior to the measurement of the rate of lipogenesis, the medium was removed, the cells washed once with 0.5ml of PBS, and the medium replaced with fresh 1ml of fresh DMEM (4.5g/l glucose, unless otherwise stated), containing 2mM glutamine and supplemented with 100 units/ml penicillin and 100 μ g/ml streptomycin (pen/strep). The rate of lipogenesis was determined by measuring the amount of 14 C-labelled D-glucose incorporated into the fatty acids of cell lipid over a 4 hour period, using a modification of the method described by Christie *et al.* (1970). 0.1 μ Ci/ml [14 C]-glucose was added to the

medium for a 4 hour period, after which time the medium was removed and the cells were lysed by the addition of GIT buffer. To obtain blank values, 0.1 μ Ci/ml [14 C]-glucose added to 3 wells containing cells for one minute before the medium was removed and the cells were lysed. Lysates were transferred to 1.5ml eppendorf tubes, and lipids were extracted by the addition of an equal volume of water-saturated chloroform. The samples were mixed well and centrifuged at 10,000g for 5 minutes in an eppendorf centrifuge (5402). The upper aqueous layer was carefully removed and kept separate for the determination of DNA content. The lipid-containing chloroform layer was transferred to a glass mini-scintillation vial and allowed to dry overnight, prior to the glucose extraction. Once the lipid was dry, 3ml of 5% H₂SO₄ ; 95% Methanol was added and the samples were mixed well, the pressure being released from the vials occasionally. The tubes were sealed with tape and were incubated at 80^oC for 2 hours, vortexing every 30 minutes. The vials were removed from the oven, and the samples were allowed to cool before being transferred to 15ml plastic centrifuge tubes. 2ml of distilled water and 2 ml of petroleum ether (40-60^oC) were added to each sample, and after vortexing, they were centrifuged at 1500 rpm for 15 minutes at 15^oC in a Beckman Benchtop centrifuge. The upper phase was transferred to a glass scintillation vial, and the extraction was repeated, combining the upper phases for the 2 extractions. The solvent was removed using a nitrogen line, then the scintillation cocktail of 1ml of toluene scintillation fluid and 4ml of EcoLite (ICN) was added to the vials. To obtain a value for total counts, 10 μ l of medium was removed from 4 wells at random and transferred to glass mini-scintillation vials. 4ml of emulsifier safe was added. The samples were counted in a Packard 1600 TR liquid scintillation analyser, and the rate of lipogenesis was expressed as μ mol glucose

incorporated / hr / mg DNA. The amount of DNA was determined, as described in section 2.2.5.4.

2.2.5.4 DNA assay

The amount of DNA present was determined according to the method of Labarca & Paigen (1980) using calf thymus DNA as the standard. The upper aqueous layer samples retained from the lipogenesis assays and ACC assay were used as the samples for the DNA assay. 25µl of each sample was added to 975µl of DNA assay buffer (2M NaCl, 0.1M NaH₂PO₄), and the standard was made up to 1ml, with a concentration range of 0µg/ml, 0.25µg/ml, 0.5µg/ml, 1µg/ml, 2µg/ml to 5µg/ml. Standards and samples were incubated with 500µl of 3µg/ml Bisbenzidine (Fluka Chemicals, Dorset, UK) to give a final concentration of 1µg/ml Bisbenzidine. After 30 minutes, the fluorescence was read in a Perkin-Elmer LS-5 luminescence spectrometer (excitation 360nm, emission 475nm).

2.2.5.5 Acetyl CoA carboxylase (ACC) assay

Differentiation medium was removed from the cells and replaced with differentiation medium minus FCS and insulin 8 days after the induction of adipogenesis. After an overnight period, growth hormone (100ng/ml) and / or insulin (100ng/ml) were added to the medium for either 24 hours or 48 hours, as indicated in the results chapters. Following the chronic and / or acute treatment of 3T3-F442A adipocyte cells with hormones, as indicated in the results chapters, total activation and the proportion of active acetyl CoA Carboxylase (ACC), the rate-limiting enzyme in fatty acid synthesis, present in the adipocytes was determined using a modification of the method of Borland *et al.* (1994). For this purpose, the 3T3-F442A adipocytes were

grown in 6 well tissue culture plates. To prepare the samples, medium was removed from the wells and the cells were washed twice with ice-cold Phosphate Buffered Saline (PBS). 4 wells of each 6 well plate were scraped into 1ml of homogenising buffer (pH 7.4), consisting of 300mM sucrose, 30mM Tris, 1mM EDTA and 1mM GSH (reduced glutathione), using a plastic rod, and the homogenate was passed through a 26 gauge needle several times. This was then centrifuged for at 14,000 rpm for 1 minute at 4°C in an eppendorf centrifuge (5402), and the supernatant was transferred to a fresh eppendorf tube. The sample was assayed for ACC activity immediately.

To measure the initial ACC activity in each sample, 0.1M NaF was also included in the homogenising buffer. The samples were prepared one at a time, and assayed for ACC activity immediately. 150µl of each sample was transferred to a glass scintillation vial which contained 850µl of assay mix with or without acetyl CoA. The assay mix consisted of 100µl of 200mM Tris / HCl (pH 7.5), 200µl of 100mM MgCl₂, 200µl of 1mM EDTA, 100µl of 200mM citrate (pH 7.5), 50µl of 50mg/ml BSA, 10µl of 100mM GSH, 100µl of 75mM ATP (pH 7.5), 25µl of sodium[¹⁴C]bicarbonate (2µCi/ml NaH¹⁴CO₃ made up in 500mM sodium bicarbonate at a ratio of 1:40), 20µl of 4.25M sodium fluoride, plus or minus 50µl of 3.6mg/ml acetyl CoA, and made up to 850µl with distilled water. The (+) acetyl CoA samples were done in triplicate and the (-) acetyl CoA samples were done individually. The assay mixes were stoppered in the glass scintillation vials, and stored at 4°C until immediately prior to the assay, then only the vials required for one homogenate were placed in a water bath at 37°C. The homogenate was prepared as described above, and 150µl was transferred to each

vial for 90 seconds. The reaction was stopped by adding 200 μ l of 6N HCl to each vial, after which time the vials were transferred to a heating block at 65 $^{\circ}$ C for 75 minutes. After the samples had cooled, 10mls of emulsifier safe was added to each vial, and the samples were counted in a Packard 1600 TR liquid scintillation analyser.

To measure the total ACC activity in each sample, the homogenates were prepared as described above, but no NaF was present in the homogenising buffer and the samples were pre-incubated in assay mix at 37 $^{\circ}$ C prior to addition of acetyl CoA. The amount of ACC activity was expressed as picomole / minute / mg DNA. The amount of DNA was determined, as described in section 2.2.5.4.

2.2.6 Determination of mean cell volume and number of adipocytes per gram of tissue

Rat or ovine tissue explants were prepared as described earlier (sections 2.2.1.1 and 2.2.1.2). The explants were added to 5ml of a collagenase digest mix of 1mg/ml collagenase (type II) and 40 mg/ml BSA made up in Medium 199, and were left at 37 $^{\circ}$ C for between 5 and 7 hours, without shaking (to minimise disruption of the cells). After passing through a sieve, samples (5 μ l) of cell suspension were transferred to a warm haemocytometer, and the diameter of 100 cells was measured using a Projectina microscope at X 145 magnification. An estimate of the number of cells per gram tissue was obtained by dividing the total lipid content of the tissue (see section 2.2.7) by the mean cell volume and multiplied by lipid density (taken to be 0.915).

2.2.7 Determination of lipid content of ovine and rat adipose tissue (Folch

Extraction)

Rat or ovine tissue explants were prepared as described earlier (sections 2.2.1.1 and 2.2.1.2). Tissue pieces were blotted and weighed, before being added to 30 ml glass centrifuge tubes containing 2 drops of 1N HCl and 8ml of chloroform / methanol (1:1). The tubes were shaken and left at room temperature overnight, after which time 4ml of chloroform was added. The tubes were shaken again and left for a minimum period of 2 hours. 3ml of 0.88% KCl was then added to the tubes and the tubes were shaken again before being centrifuged at 2500 rpm for 15 minutes. The upper phase was removed and the lower phase was taken to dryness using a rotary evaporator. The remaining lipid was then re-dissolved in 3ml of chloroform, filtered into a weighed glass scintillation vial and placed on a heating block at 65°C under an airline to remove the chloroform. After the vials had been left to cool they were weighed again. The difference in weight of the vial gave the lipid weight.

2.2.8 Statistical analysis

Each group of observations was obtained from a different animal (rats and sheep). Each observation was obtained from 3 replicates. Results are presented as means +/- S.E.M. and statistical analysis was by Student's t-test for either paired or unpaired observations, using Analysis of Variance (ANOVA) or REsidual Maximum Likelihood (REML ; Patterson & Thompson, 1971), as indicated in the text.

CHAPTER THREE

THE EFFECT OF TUMOUR NECROSIS FACTOR

ALPHA (TNF α) ON LIPOLYSIS AND

LIPOGENESIS IN OVINE ADIPOSE TISSUE

IN VITRO

3.1 Introduction

Lipolysis is under complex and acute endocrine control. The rate-limiting enzyme hormone-sensitive lipase (HSL) can be phosphorylated and activated by cAMP-dependent protein kinase A (PKA), so changes in adenylate cyclase (AC) activity are closely connected with the control of lipolysis (Belfrage, 1985). Growth hormone (GH) is thought to alter lipolysis *via* indirect mechanisms by altering the maximum response or sensitivity of adipocytes to acutely acting lipolytic or antilipolytic agents.

Chronic treatment with bovine GH *in vivo* increases the lipolytic response of adipose tissue to catecholamines *in vitro* in rats (Vernon *et al.*, 1987) and sheep, and *in vivo* in cows (McCutcheon and Bauman, 1986; Peters, 1986; Sechen *et al.*, 1990). Chronic treatment with bovine GH *in vivo* or *in vitro* reduces the response to the antilipolytic effects of the adenosine analogue PIA in rat (Vernon *et al.*, 1987; Doris *et al.*, 1994) and bovine (Lanna *et al.*, 1992) adipose tissue *in vitro*. The mechanism whereby GH chronically regulates lipolysis and lipogenesis is not resolved. Studies by Borland *et al.* (1994) have implicated a protein with a very short half-life that is possibly a secreted protein.

The cytokine Tumour Necrosis Factor alpha (TNF α) is produced and secreted by adipocytes. It has been published by Green *et al.* (1994) that TNF α chronically causes an increase in lipolysis in rat epididymal adipocytes like that of GH. This raised the possibility that TNF α might be this putative secreted protein, so initial studies were carried out comparing the effect of GH and TNF α on lipolysis and also lipogenesis in ovine and rat adipose tissue explants.

3.2 Experimental procedure

3.2.1 Lipolysis assay : ovine and rat adipose tissue explants

Ovine subcutaneous or popliteal adipose tissue explants or rat adipose tissue explants were prepared, as described in sections 2.2.1.1 and 2.2.1.2, and cultured in Medium 199 for 24 hours in 24 well plates. The explants were then transferred to fresh Medium 199 that either had no additions (con), growth hormone added (gh ; 100ng/ml) or Tumour Necrosis Factor alpha added (TNF α (murine, unless stated otherwise) ; 20ng/ml or 50ng/ml, as indicated). These were left at 37°C for a further 24 hours, after which time the tissue was transferred to 2.5mls of Ringer's bicarbonate medium (+ 3% BSA + 1mg/ml glucose + 2mM sodium acetate + 25mM Hepes) for 3 hours at 37°C. This contained either no additions (basal), + adenosine deaminase (ad ; 8 μ g/ml), ad + isoproterenol (isop ; 10⁻⁵M) or ad + isop + PIA (100nM, unless otherwise indicated). After the 3 hour incubation period, 1ml of medium was removed and assayed for glycerol content, as described in section 2.2.3.2.

3.2.2 Lipogenesis assay : ovine adipose tissue explants

Ovine adipose tissue explants were prepared, as described in section 2.2.1.1, and cultured in Medium 199 for 24 hours in 24 well plates. The explants were then transferred to fresh Medium 199 that either had no additions (con), growth hormone added (gh ; 100ng/ml), insulin added (ins ; 100ng/ml) or murine Tumour Necrosis Factor alpha added (TNF α ; 20ng/ml or 50ng/ml, as indicated). These were left at 37°C for a further 24 hours, after which time the tissue was transferred to 2.5mls Medium 199 containing 0.1 μ Ci/ml of [¹⁴C]-sodium acetate to give a final concentration of 2.6mM acetate. After a 2 hour period at 37°C, the tissue was

removed from the medium, and the rate of lipogenesis was assessed, as described in section 2.2.3.3.

3.2.3 Statistical analysis

Results are expressed as the means \pm S.E.M., and statistical analysis was by analysis of variance (ANOVA), unless stated otherwise.

3.3 Results

3.3.1 Effects of growth hormone on lipolysis in ovine subcutaneous adipose tissue

The lipolytic effects of GH were determined in primary cultures of ovine subcutaneous adipose tissue. After 48 hours incubation, there was an increase in the rate of lipolysis in both unstimulated and catecholamine-stimulated tissue explants, as shown in Figure 3.1. Analysis of variance shows that GH significantly increased the rate of lipolysis overall, also shown in Figure 3.1 ($P < 0.05$). GH alone had a tendency towards causing an increase in the rate of lipolysis, which is in accordance with previous findings (Vernon *et al.*, 1987 ; Watt *et al.*, 1991).

The experiment shown in Figure 3.2 indicates that GH also has a tendency to diminish the inhibition effect of PIA on lipolysis, as found previously (Vernon *et al.*, 1991b; Doris *et al.*, 1994).

Effect of Growth Hormone on basal and stimulated lipolysis in ovine subcutaneous adipose tissue

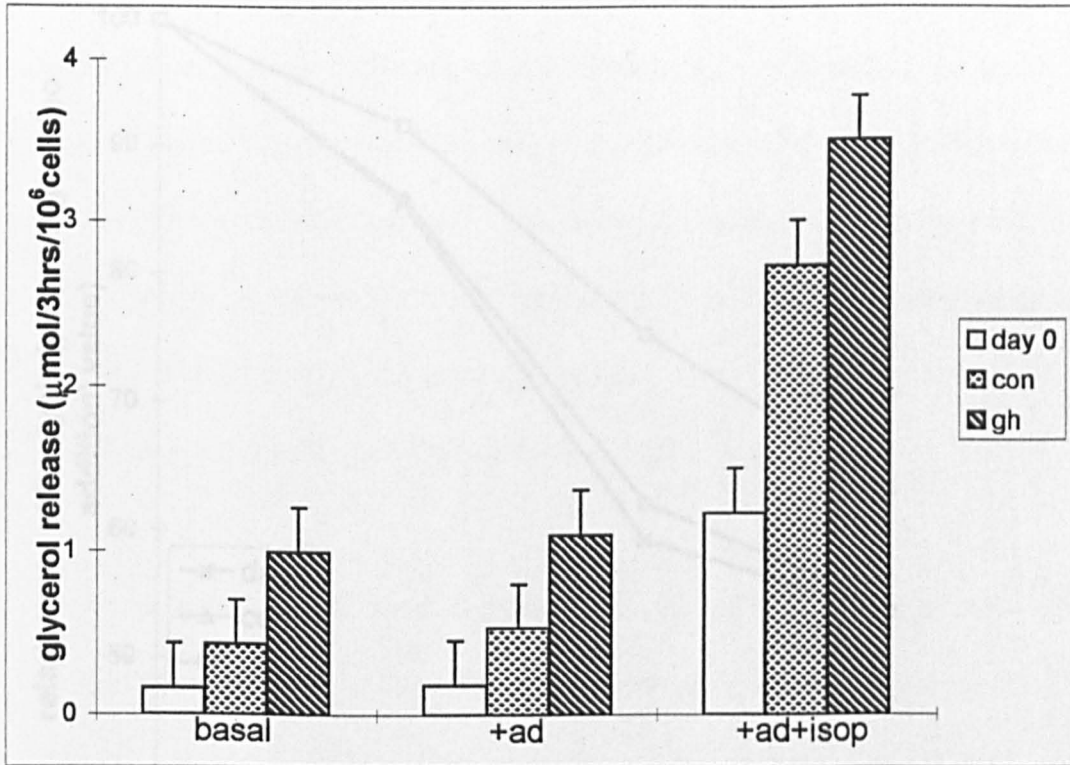


Figure 3.1. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, Growth Hormone (gh ; 100ng/ml) was added for a further 24 hour period prior to the measurement of the rate of lipolysis (con = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with either no addition (basal), adenosine deaminase (ad ; 8μg/ml) or ad + isoproterenol (isop ; 10⁻⁵M). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. Day 0 tissue was assayed at the time the tissue was removed from the sheep. Results are the means of 8 observations, + S.E.M.

Effect of Growth Hormone on the inhibition of lipolysis by PIA in ovine subcutaneous adipose tissue

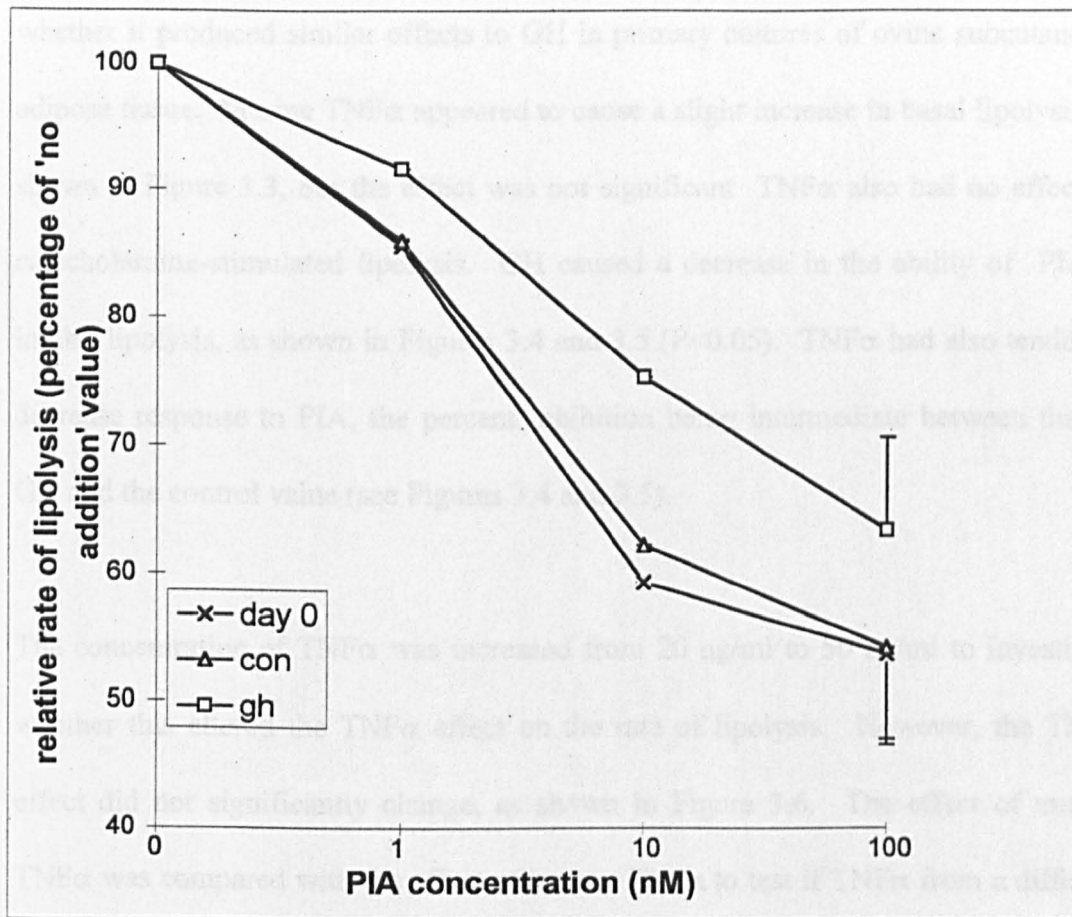


Figure 3.2. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, Growth Hormone (gh : 100ng/ml) was added for a further 24 hours prior to the measurement of the rate of lipolysis (con = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with adenosine deaminase (8 μ g/ml) + isoproterenol (10⁻⁵M) + various concentrations of PIA, as indicated. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Day 0 tissue was assayed at the time the tissue was removed from the sheep. Results are the means of 6 observations, \pm S.E.M.

3.3.2 Effects of tumour necrosis factor alpha (TNF α) on lipolysis in ovine

subcutaneous adipose tissue

The chronic effects of the cytokine, TNF α , on lipolysis were investigated to determine whether it produced similar effects to GH in primary cultures of ovine subcutaneous adipose tissue. Murine TNF α appeared to cause a slight increase in basal lipolysis, as shown in Figure 3.3, but the effect was not significant. TNF α also had no effect on catecholamine-stimulated lipolysis. GH caused a decrease in the ability of PIA to inhibit lipolysis, as shown in Figures 3.4 and 3.5 ($P < 0.05$). TNF α had also tended to decrease response to PIA, the percent inhibition being intermediate between that of GH and the control value (see Figures 3.4 and 3.5).

The concentration of TNF α was increased from 20 ng/ml to 50 ng/ml to investigate whether this altered the TNF α effect on the rate of lipolysis. However, the TNF α effect did not significantly change, as shown in Figure 3.6. The effect of murine TNF α was compared with the effect of human TNF α to test if TNF α from a different species had a greater effect on the rate of ovine lipolysis. Figure 3.7 shows that there was no obvious difference between the two TNF α from different species.

3.3.3 Hormone effects on lipolysis measured at two regions in ovine popliteal

adipose tissue

Studies on ovine popliteal adipose tissue explants were undertaken to investigate if the distance from the lymph node caused variation in the effect of TNF α on the rate of lipolysis (see introduction, chapter 1, section 1.4.1). TNF α had no effect on basal, catecholamine-stimulated or PIA-inhibited lipolysis in adipose tissue from "near"

Effect of Growth Hormone and $\text{TNF}\alpha$ on lipolysis in ovine subcutaneous adipose tissue

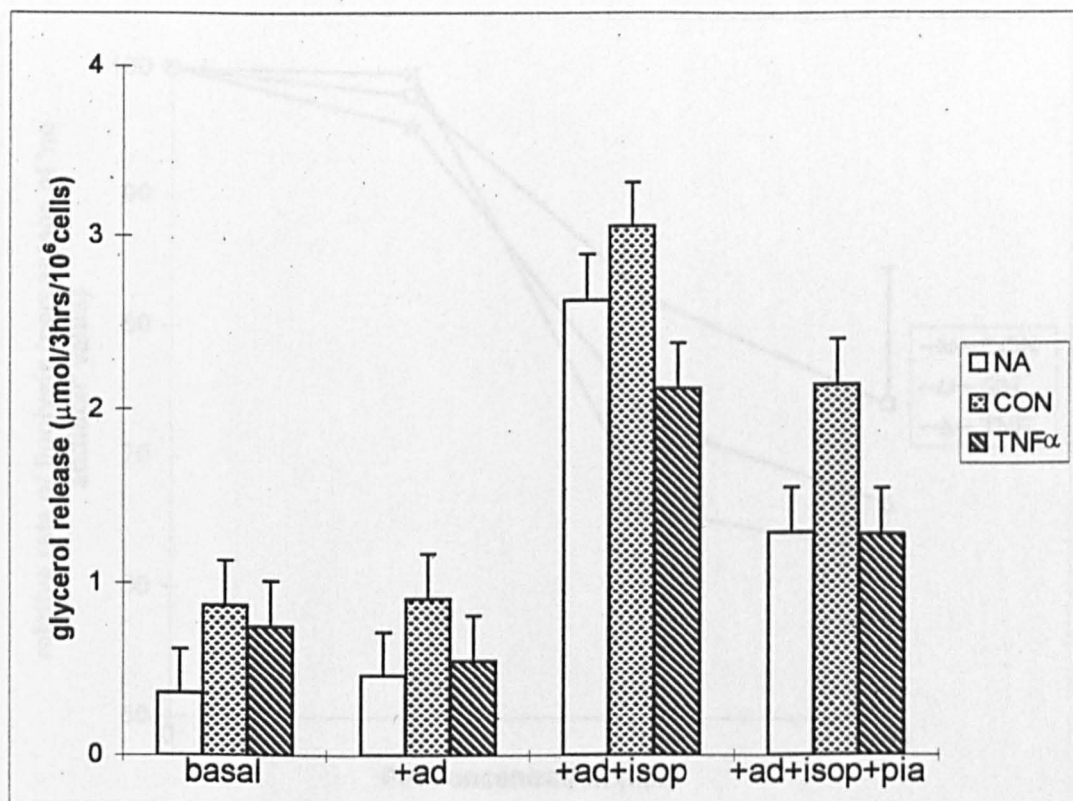


Figure 3.3. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml) or murine $\text{TNF}\alpha$ (20ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with either no additions (basal), adenosine deaminase (ad ; 8 $\mu\text{g/ml}$) +/- isoproterenol (isop ; 10⁻⁵M) +/- PIA (100nM), as shown. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 7 observations + S.E.M.

Effect of Growth Hormone and $\text{TNF}\alpha$ on the inhibition of lipolysis by PIA in ovine subcutaneous adipose tissue

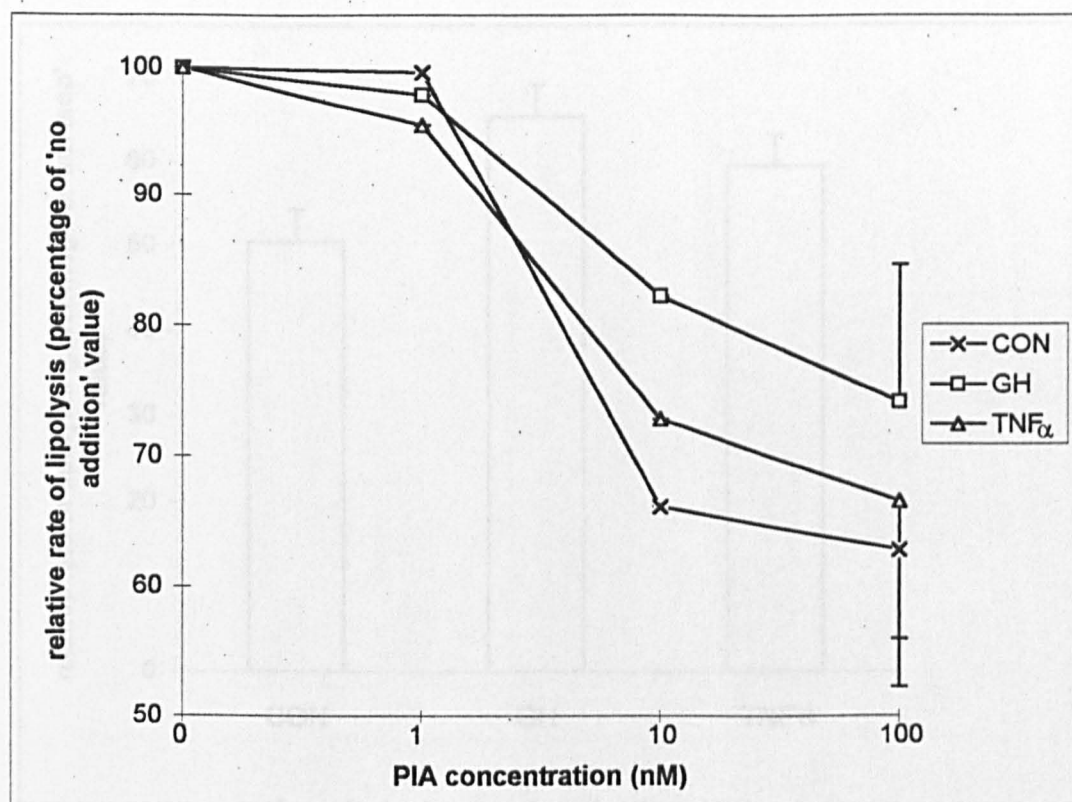


Figure 3.4. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml) or murine $\text{TNF}\alpha$ (20ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with adenosine deaminase (ad ; 8 μ g/ml) + isoproterenol (isop ; 10⁻⁵M) + various concentrations of PIA, as indicated. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 4 observations, +/- S.E.M.

**Effect of Growth Hormone and $\text{TNF}\alpha$ on the inhibition of lipolysis
by PIA in ovine subcutaneous adipose tissue**

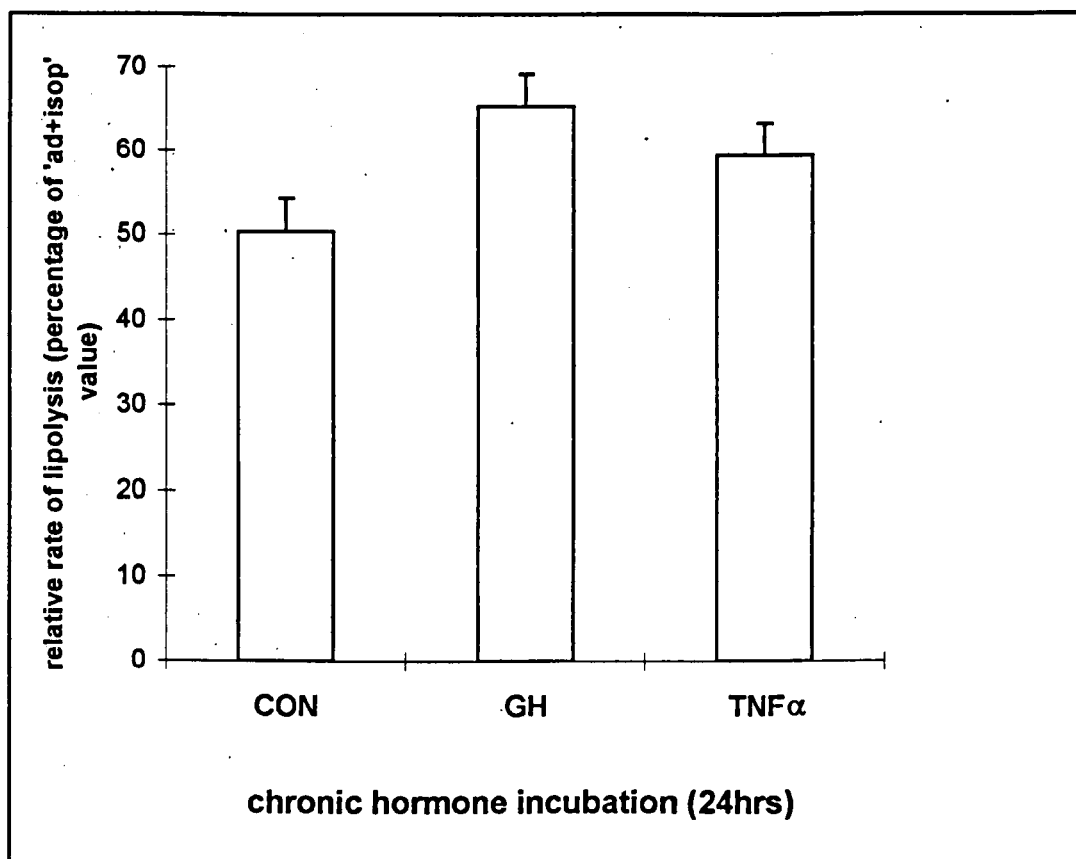


Figure 3.5. Adipose tissue was removed from ovine subcutaneous tissue and explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml), or murine $\text{TNF}\alpha$ (20ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, containing either adenosine deaminase (8 $\mu\text{g}/\text{ml}$) + isoproterenol (10^{-5}M) or ad + isop + PIA (100nM). After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 7 observations + S.E.M.

Effect of Growth Hormone and different $\text{TNF}\alpha$ concentrations on lipolysis in ovine subcutaneous adipose tissue

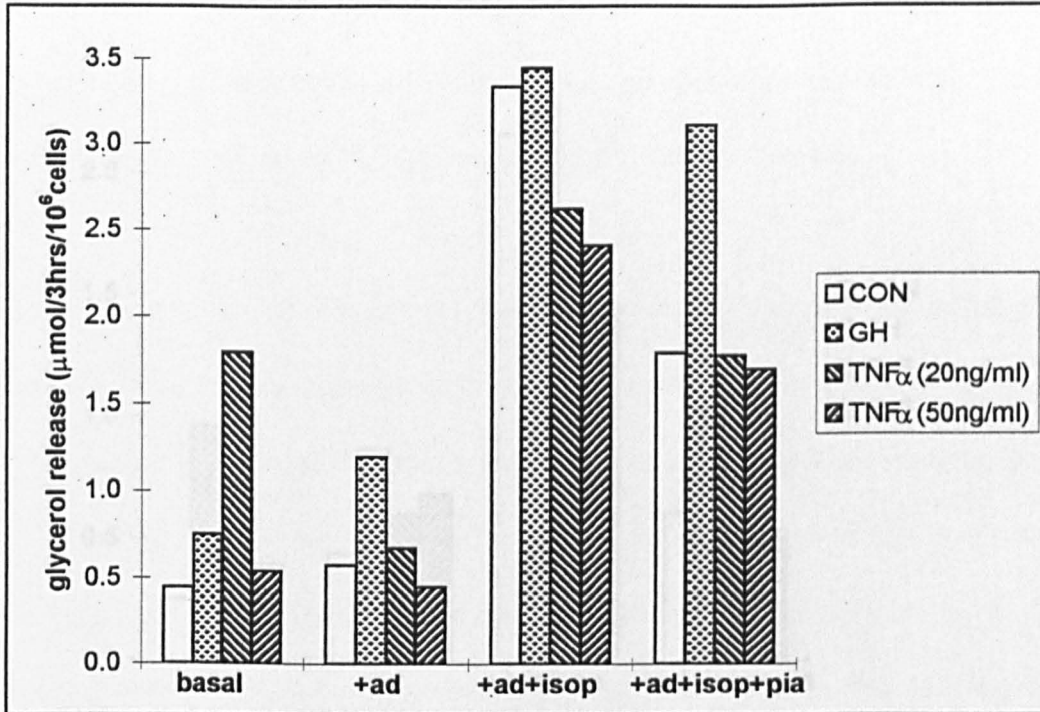


Figure 3.6. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml) or murine $\text{TNF}\alpha$ (20ng/ml or 50ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON - control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium with either no additions (basal), adenosine deaminase (ad ; 8 μ g/ml) +/- isoproterenol (isop ; 10⁻⁵M) +/- PIA (100nM), as shown. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 2 observations.

Murine TNF α vs human TNF α ; effects on lipolysis in ovine subcutaneous adipose tissue

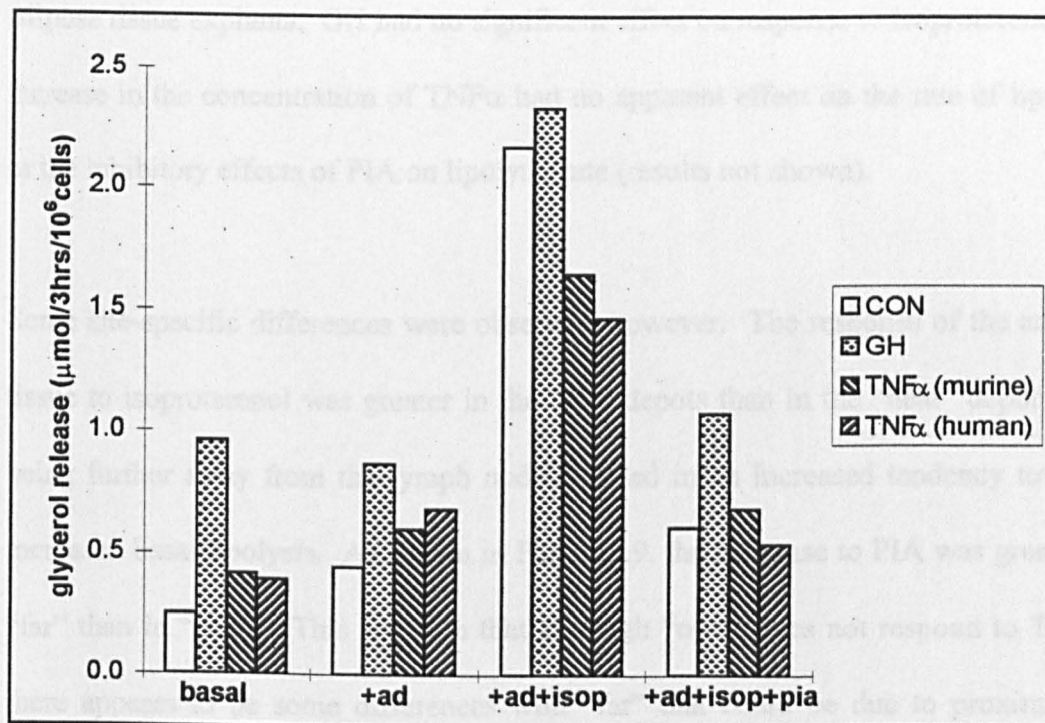


Figure 3.7. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml), murine-derived TNF α (20ng/ml) or human-derived TNF α (20ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with adenosine deaminase (8μg/ml) +/- isoproterenol (isop ; 10⁻⁵M) +/- PIA (100nM), as shown. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 2 observations.

(10mm or less) or “far” (greater than 10mm) from the lymph node, as is shown in Figure 3.8. In contrast, growth hormone increased basal lipolysis in both “near” and “far” adipose tissue, and decreased the response to PIA in “far” but not “near” ovine adipose tissue explants. GH had no significant effect on response to isoproterenol. An increase in the concentration of $\text{TNF}\alpha$ had no apparent effect on the rate of lipolysis or the inhibitory effects of PIA on lipolytic rate (results not shown).

Some site-specific differences were observed, however. The response of the adipose tissue to isoproterenol was greater in the “far” depots than in the “near” depots, and being further away from the lymph node resulted in an increased tendency towards increased basal lipolysis. As shown in Figure 3.9, the response to PIA was greater in “far” than in “near”. This suggests that although “near” does not respond to $\text{TNF}\alpha$, there appears to be some differences with “far” that could be due to proximity to lymph nodes.

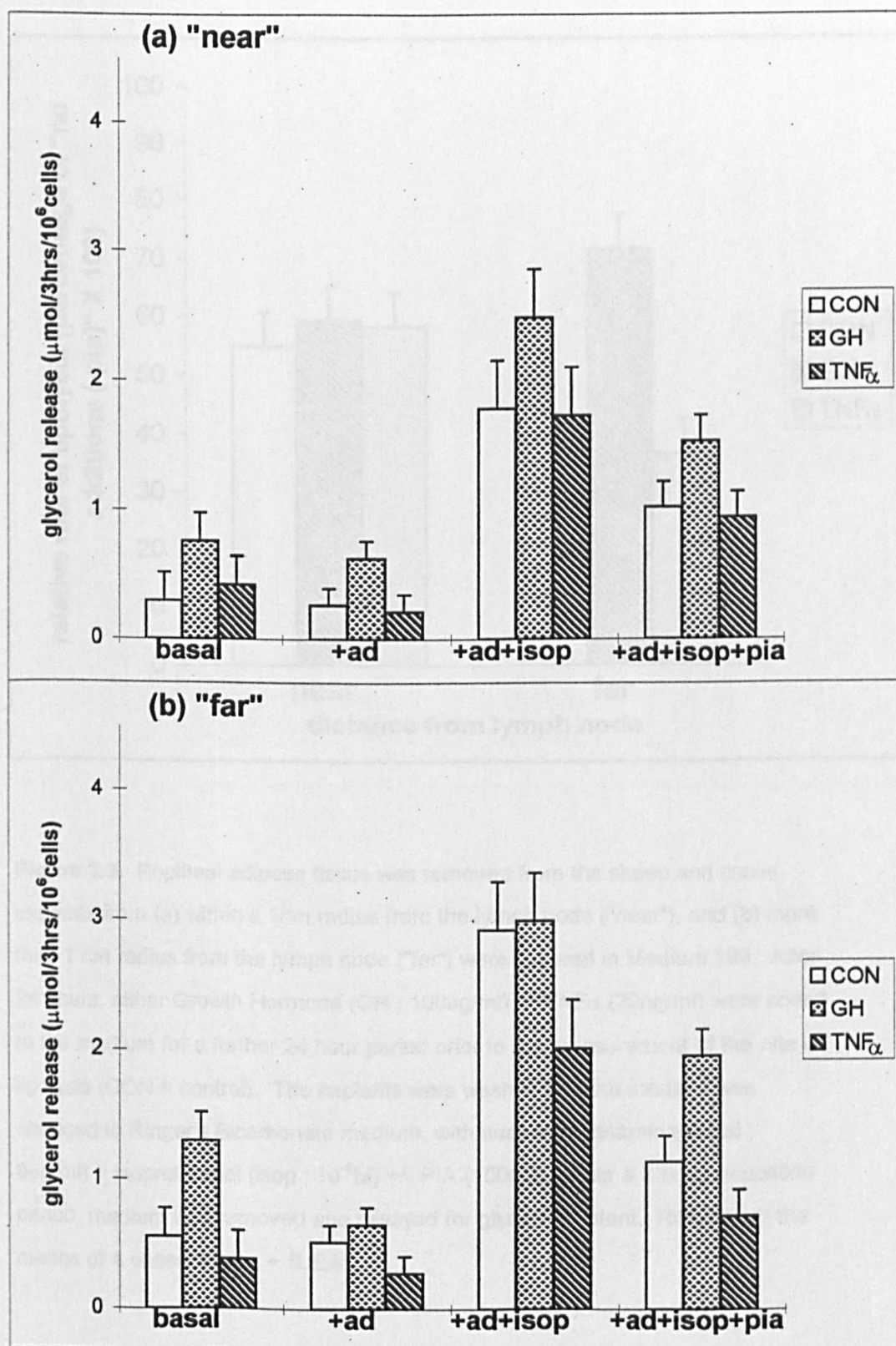
3.3.4 Effects of growth hormone and $\text{TNF}\alpha$ on lipolysis in rat adipose tissue

explants

The chronic effects of both GH and $\text{TNF}\alpha$ on lipolysis in rat adipose tissue explants were investigated. After 48 hours incubation, there was an increase in the rate of lipolysis in both unstimulated and catecholamine-stimulated primary tissue explants, as shown in Figure 3.10. Neither hormone had a significant effect on the rate of lipolysis. Figure 3.11 shows that both GH and $\text{TNF}\alpha$ tended to diminish the inhibitory effect of the adenosine analogue, PIA, on lipolytic rate ($P=0.07$ for $\text{TNF}\alpha$).

Figure 3.8. Popliteal adipose tissue was removed from the sheep and tissue explants from (a) within a 1cm radius from the lymph node ("near"), and (b) more than 1 cm radius from the lymph node ("far") were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml) or $\text{TNF}\alpha$ (20ng/ml) were added to the medium for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with either no additions (basal), adenosine deaminase (ad ; 8 $\mu\text{g}/\text{ml}$) +/- isoproterenol (isop ; 10^{-5}M) +/- PIA (100nM), as shown. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 4 observations, + S.E.M.

Effect of Growth Hormone and $\text{TNF}\alpha$ on lipolysis in ovine
popliteal adipose tissue



Effect of Growth Hormone and $\text{TNF}\alpha$ on the inhibition of lipolysis in ovine popliteal adipose tissue

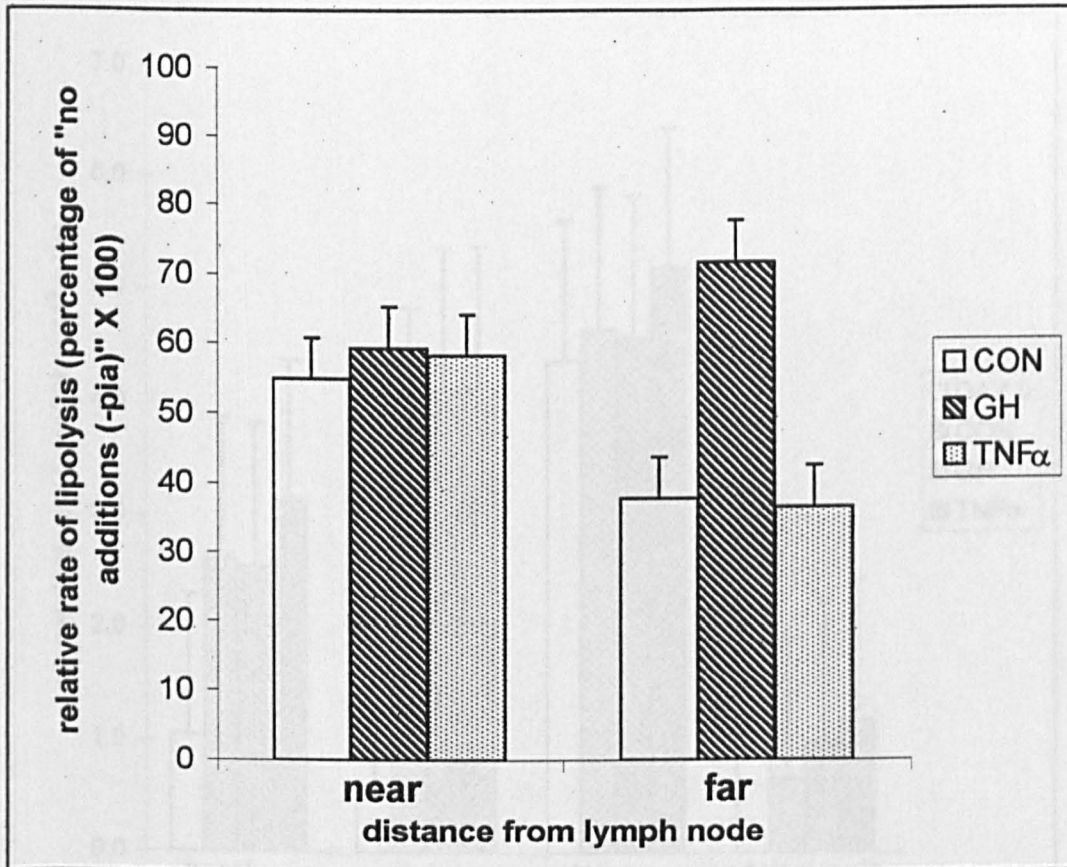


Figure 3.9. Popliteal adipose tissue was removed from the sheep and tissue explants from (a) within a 1cm radius from the lymph node ("near"), and (b) more than 1 cm radius from the lymph node ("far") were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml) or $\text{TNF}\alpha$ (20ng/ml) were added to the medium for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, with adenosine deaminase (ad ; 8 $\mu\text{g/ml}$) + isoproterenol (isop ; 10^{-5}M) +/- PIA (100nM). After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 4 observations, + S.E.M.

Effect of Growth Hormone and TNF α on lipolysis in rat

adipose tissue

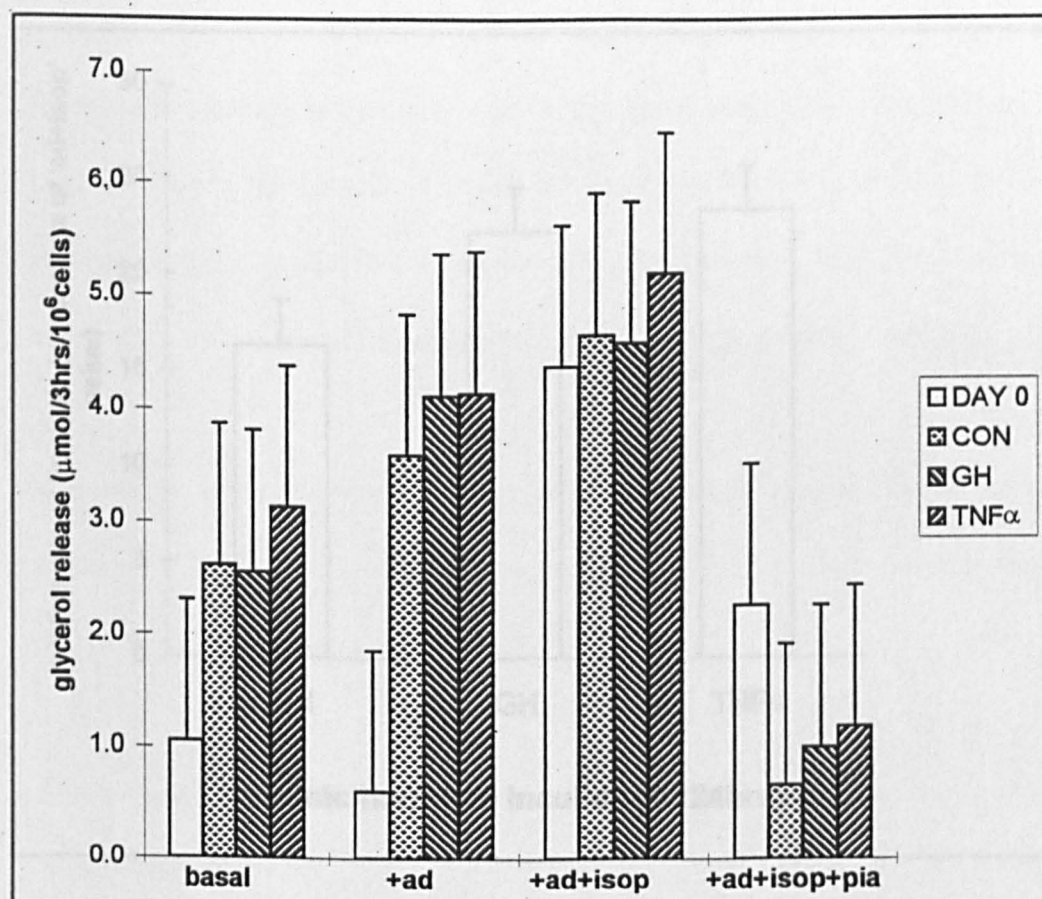


Figure 3.10. Adipose tissue was removed from the rat fat pads and tissue explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml), or murine TNF α (20ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, containing either no additions (basal), adenosine deaminase (ad ; 8 μ g/ml) +/- isoproterenol (isop ; 10⁻⁵M) +/- PIA (100nM), as indicated. After a 3 hour incubation period, medium was removed and assayed for glycerol content. Day 0 tissue was assayed at the time the tissue was removed from the rat. Results are the means of 6 observations + S.E.M.

**Effect of Growth Hormone and $\text{TNF}\alpha$ on the inhibition of lipolysis
by PIA in rat adipose tissue**

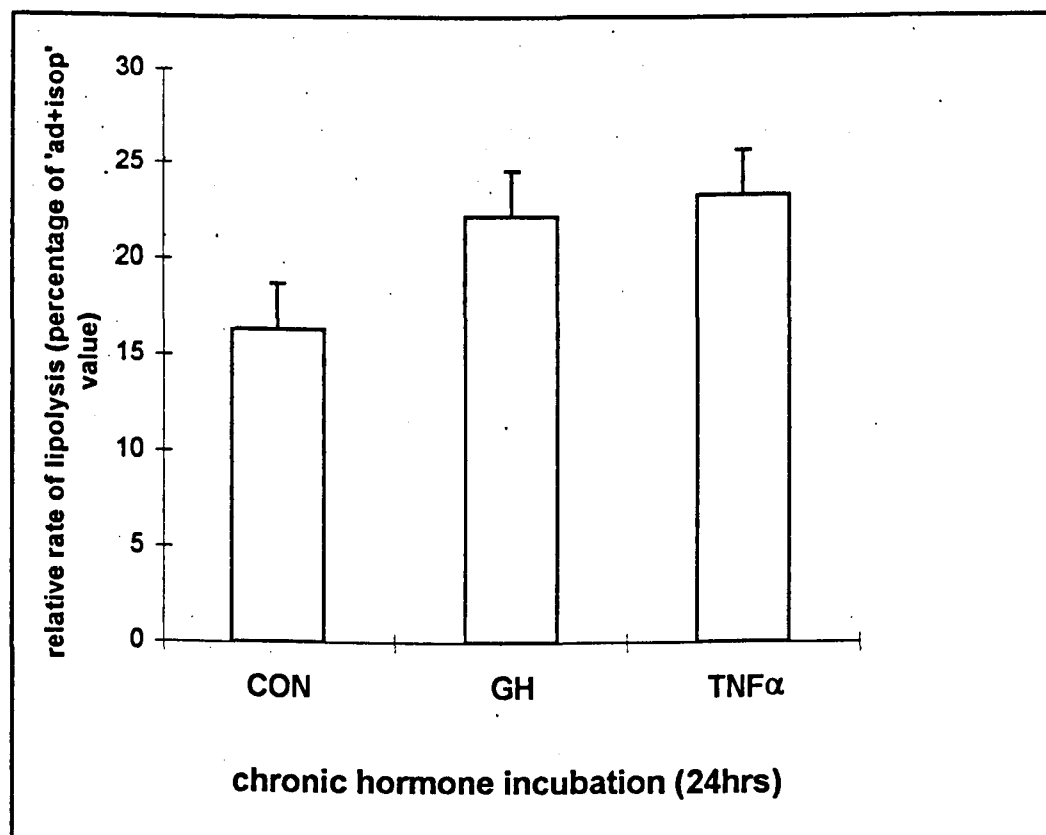


Figure 3.11. Adipose tissue was removed from the rat fat pads and tissue explants were cultured in Medium 199. After 24 hours, either Growth Hormone (GH ; 100ng/ml), or murine $\text{TNF}\alpha$ (20ng/ml) were added for a further 24 hour period prior to the measurement of the rate of lipolysis (CON = control). The explants were washed and the medium was changed to Ringer's Bicarbonate medium, containing either adenosine deaminase (8 $\mu\text{g}/\text{ml}$) + isoproterenol (10^{-5}M) or ad + isop + PIA (100nM). After a 3 hour incubation period, medium was removed and assayed for glycerol content. Results are the means of 6 observations + S.E.M.

3.3.5 Hormone effects on the rate of lipogenesis in ovine subcutaneous adipose

tissue explants

Studies on ovine subcutaneous adipose tissue explants were undertaken to investigate the chronic effects on GH, insulin and $\text{TNF}\alpha$ on lipogenesis. Figure 3.12 shows that GH caused a reduction in lipogenic rate, while chronic exposure to insulin resulted in a large increase in lipogenesis. Chronic incubation with $\text{TNF}\alpha$ alone and $\text{TNF}\alpha$ plus GH together did not alter the lipogenic rate significantly. However, inclusion of $\text{TNF}\alpha$ with insulin resulted in a reduction ($P < 0.01$) in lipogenesis (see Figure 3.12).

There was no apparent different effect on the rate of lipogenesis in the absence or presence of GH or insulin when the concentration of $\text{TNF}\alpha$ was increased from 20 ng/ml to 50 ng/ml (results not shown).

3.3.6 Hormone effects on lipogenesis in subcutaneous adipose tissue explants of

lactating sheep

The effects of dexamethasone, insulin and $\text{TNF}\alpha$ were investigated on the rate of lipogenesis in subcutaneous adipose tissue explants from lactating sheep, after both 24 hours and 48 hours incubation of the hormones with the tissue explants. GH abolishes the effects of insulin and dexamethasone after a 48 hour period, so this study was to determine if $\text{TNF}\alpha$ had a similar effect. After 24 hours incubation with insulin and dexamethasone, there was a slight increase in the rate of lipogenesis, as shown in Figure 3.13. $\text{TNF}\alpha$ had no effect on lipogenesis by itself, and it did not reduce stimulation of lipogenesis by insulin and dexamethasone. After a further 24 hours incubation, there was a much greater increase ($P < 0.001$) in lipogenesis in the

presence of insulin and dexamethasone. Again, there was no effect on lipogenic rate by the presence of $\text{TNF}\alpha$. However, $\text{TNF}\alpha$ did cause inhibition of the stimulation of lipogenesis by insulin and dexamethasone ($P < 0.001$), reducing the level to half that seen in the absence of $\text{TNF}\alpha$.

**Effect of Growth Hormone, Insulin and $\text{TNF}\alpha$ on lipogenesis in
ovine subcutaneous adipose tissue**

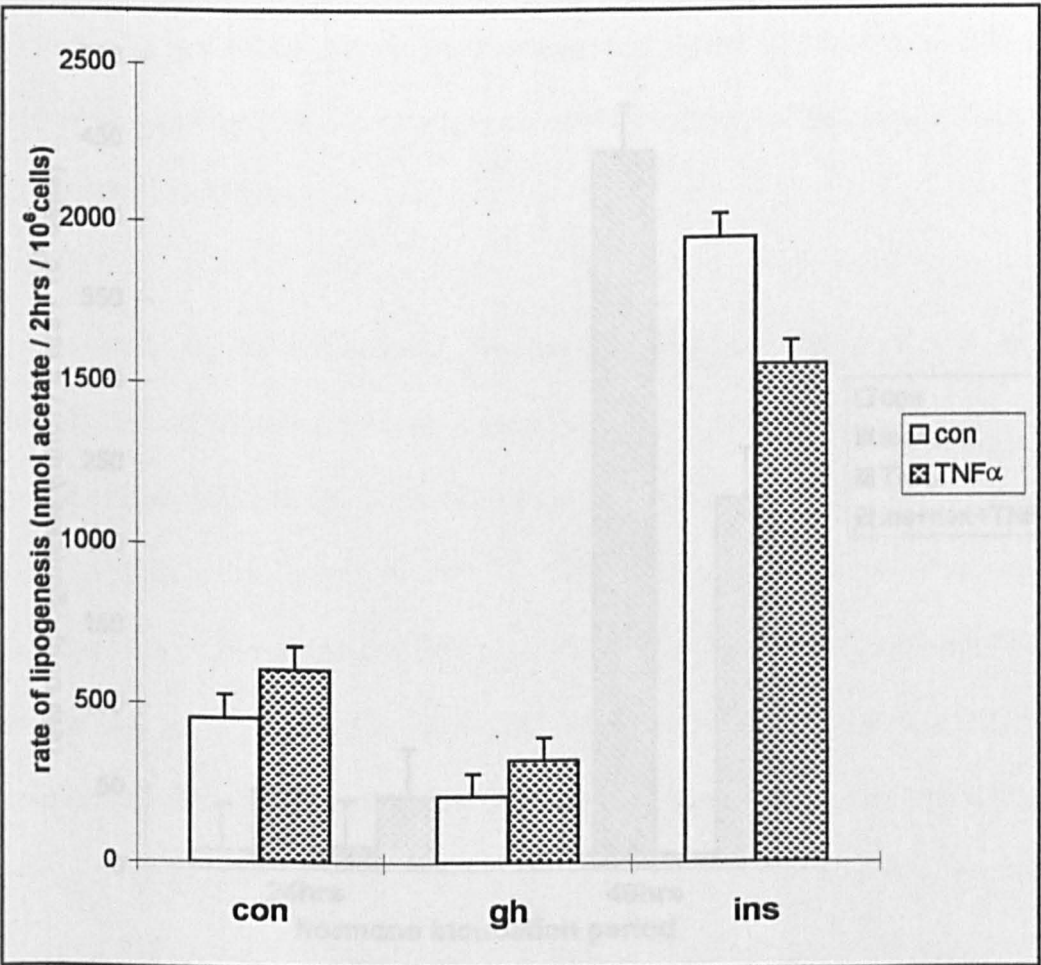


Figure 3.12. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, the medium was replaced by fresh medium, with either no additions (con), Growth Hormone (gh ; 100ng/ml), Insulin (ins ; 100ng/ml) added +/- $\text{TNF}\alpha$ (20ng/ml). These hormones were present in the medium for 24 hours prior to the measurement of the rate of lipogenesis. This was determined by measuring the amount of ^{14}C -labelled sodium acetate incorporated into the tissue explants over a 2 hour period. Results are the means of 5 observations, + S.E.M.

Effect of Dexamethasone, Insulin and $\text{TNF}\alpha$ on lipogenesis in subcutaneous adipose tissue of lactating sheep

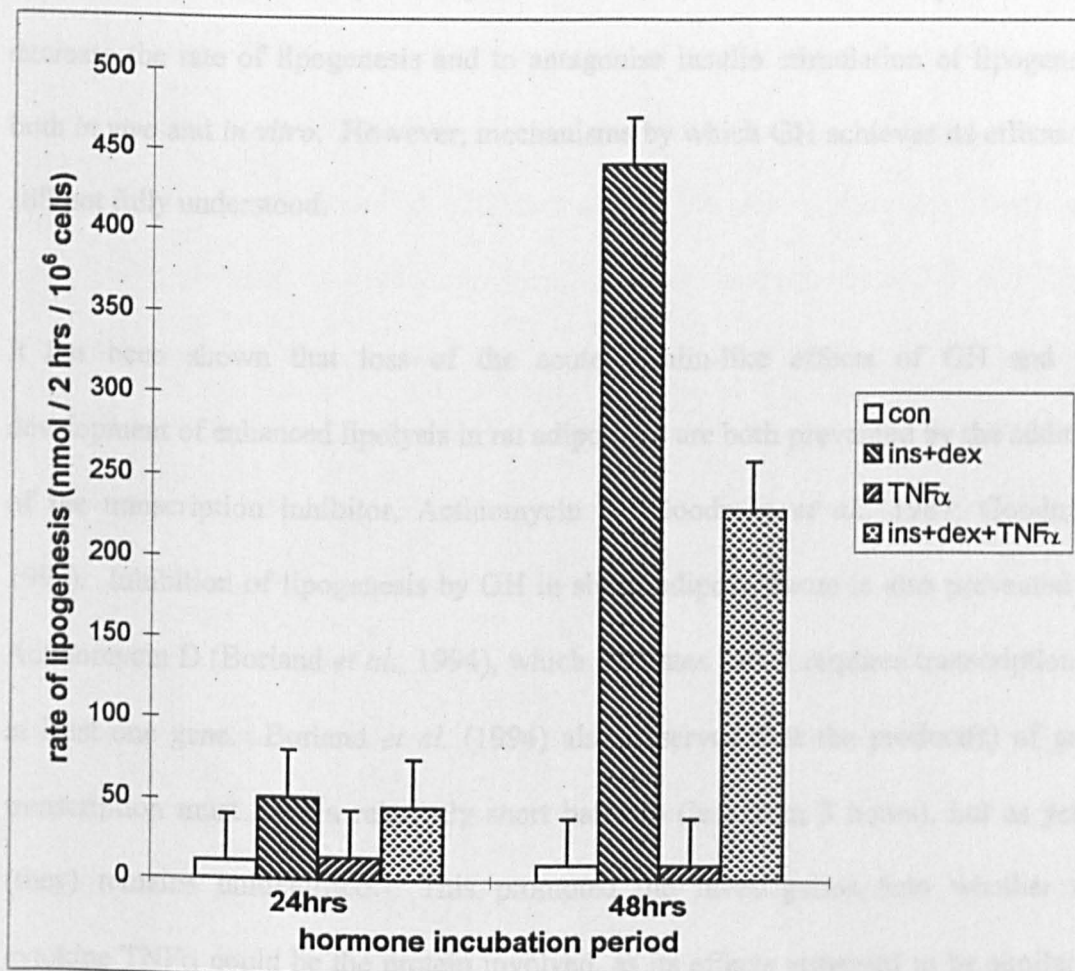


Figure 3.13. Subcutaneous adipose tissue was removed from the sheep and tissue explants were cultured in Medium 199. After 24 hours, the medium was replaced by fresh medium, with either no additions (con), dexamethasone (dex ; 100ng/ml) and/or Insulin (ins ; 100ng/ml) and/or $\text{TNF}\alpha$ (20ng/ml) added. These hormones were present in the medium for either 24 or 48 hours prior to the measurement of the rate of lipogenesis. This was determined by measuring the amount of ^{14}C -labelled sodium acetate incorporated into the tissue explants over a 2 hour period. Results are the means of 4 observations + S.E.M.

3.4 Discussion

GH has acute, insulin-like effects and chronic insulin-antagonistic effects on adipose tissue. GH acts chronically on adipose tissue to enhance the rate of lipolysis and to decrease the rate of lipogenesis and to antagonise insulin stimulation of lipogenesis both *in vivo* and *in vitro*. However, mechanisms by which GH achieves its effects are still not fully understood.

It has been shown that loss of the acute insulin-like effects of GH and the development of enhanced lipolysis in rat adipocytes are both prevented by the addition of the transcription inhibitor, Actinomycin D (Goodman *et al.*, 1987; Goodman, 1993). Inhibition of lipogenesis by GH in sheep adipose tissue is also prevented by Actinomycin D (Borland *et al.*, 1994), which indicates that it requires transcription of at least one gene. Borland *et al.* (1994) also observed that the product(s) of gene transcription must have a relatively short half-life (less than 3 hours), but as yet it (they) remains unidentified. This prompted the investigation into whether the cytokine TNF α could be the protein involved, as its effects appeared to be similar to those of GH on adipose tissue metabolism (Green *et al.*, 1994; Gasic *et al.*, 1999).

Green and colleagues found that incubating primary rat epididymal adipocytes with TNF α resulted in a 2.7-fold increase in the rate of basal lipolysis (Green *et al.*, 1994). This effect required a minimum of 6-12 hours to become apparent, and was maximal by 24 hours incubation, which is comparable with the time course observed for GH to increase lipolysis (Gorin *et al.*, 1990). I found that TNF α incubation with the primary ovine subcutaneous tissue explants had no effect on either basal lipolysis or

catecholamine-stimulated lipolysis in ovine subcutaneous tissue, whereas GH had a tendency to increase lipolysis in both stimulated and unstimulated explants, as seen previously (e.g. Doris *et al.*, 1994). Culture with GH also resulted in a decrease in response to PIA. $\text{TNF}\alpha$ appears to attenuate the effect of PIA on sheep adipose tissue, but the effect is smaller than that of GH. Green and colleagues had found that $\text{TNF}\alpha$ had an attenuating effect on PIA inhibition of lipolysis in rats, although no significant effect was observed on isoproterenol-stimulated lipolysis (Gasic *et al.*, 1999). Chronic incubation with rat epididymal explant tissue showed that after 48 hours incubation, either in the presence or absence of additions, the rate of lipolysis was generally greatly increased, and so differences due to $\text{TNF}\alpha$ and GH were difficult to detect. Rat adipose tissue shows a much greater stimulation of lipolysis by adenosine deaminase (removal of adenosine) than is observed in ovine adipose tissue. Both GH and $\text{TNF}\alpha$ (consistent with Gasic *et al.*, 1999) had a tendency to reduce the response to PIA in rat explants, but the effect was not great. Overall, $\text{TNF}\alpha$ is less effective in changing lipolysis in sheep adipocytes than GH.

Pond and colleagues have previously demonstrated that, in guinea pigs, adipose tissue around lymph nodes has properties which enables it to participate in local interactions with lymph node lymphoid cells (Pond & Mattacks, 1995; Mattacks & Pond, 1997). Lipolysis increased more than 3-fold in adipocytes near to the node when cultured with lymph node lymphoid cells *in vitro*, whereas lipolysis from adipose tissue further away from the node in the same depot barely doubled. Almost all lymph nodes are embedded in adipose tissue (Yoffey *et al.*, 1970) which is protected from the general mobilisation of fat reserves observed during starvation or fever (Pond, 1996a,b), and

most peripheral adipose tissue depots contain at least one lymph node (Pond, 1996a). Both the lipolytic response and fatty acid composition of triacylglycerols from popliteal adipose tissue varies with distance from the lymph nodes in guinea pigs (Pond, 1996a). Cytokines often mediate interactions between lymphoid cells and the immune system and other cell types. $\text{TNF}\alpha$ is a cytokine produced by many cell types, including adipocytes. Type II $\text{TNF}\alpha$ receptors are present continuously on adipocytes around the popliteal lymph node, and it has been shown that endotoxin (lipopolysaccharide) injection results in the appearance of $\text{TNF}\alpha$ type I receptors and enhanced lipolysis on adipocytes near the lymph node, but not from further away in rat adipose tissue (MacQueen & Pond, 1998; Pond & Mattacks, 1998). However, the results in this chapter indicate that there are no differences in the ability of $\text{TNF}\alpha$ to stimulate basal or catecholamine-stimulated lipolysis in adipose tissue from either near or far from the lymph node in ovine popliteal tissue explants. It has also been found that there are no changes in fatty acid composition with distance from the lymph node in popliteal adipose tissue from sheep, which is consistent with my findings (Vernon & Pond, 1997). There appear to be some differences between “near” and “far”, however. Ovine adipose tissue from “near” the lymph node showed a smaller increase in response to isoproterenol, and a diminished response to PIA. This presumably reflects the proximity to the lymph node.

Although chronic exposure to $\text{TNF}\alpha$ only appeared to result in a slight change in lipolysis in ovine and rat adipose tissue explants, I found that $\text{TNF}\alpha$ did cause a reduction in insulin stimulation of lipogenesis. $\text{TNF}\alpha$ did not appear to have any effects by itself, but it did block the effects of insulin in ovine subcutaneous adipose

tissue explants. It has previously been shown that in 3T3 adipocytes, TNF α inhibits lipoprotein lipase synthesis (Semb *et al.*, 1987), as well as ACC (Pape & Kim, 1988), fatty acid-binding protein and glycerol phosphate dehydrogenase (Torti *et al.*, 1985) and fatty acid synthase (Pekala *et al.*, 1983) synthesis. All these enzymes are involved in fat synthesis. It has been shown that the presence of TNF α results in peripheral insulin resistance in rats (Lang *et al.*, 1992) and humans (Van der Poll *et al.*, 1991). Treatment of 3T3-F442A adipocytes with TNF α leads to downregulation of GLUT4 mRNA (Stephens & Pekala, 1991) and downregulation in expression of GLUT4, the insulin stimutable glucose transporter, in rat 3T3-L1 adipocytes (Stephens & Pekala, 1991). The TNF α gene has been shown to be overexpressed in skeletal muscle in diabetic subjects (Saghizadeh *et al.*, 1996), as well as a high correlation being found to exist between levels of TNF α mRNA, body mass index and circulating insulin levels in adipose tissue of obese subjects (Kern *et al.*, 1995), which supports the role of the cytokine in the induction of a generalised state of insulin resistance. TNF α can decrease the tyrosine kinase activity of the insulin receptor (IR) (Hotamisligil *et al.*, 1996), and treatment of cultured murine adipocytes with TNF α induces serine phosphorylation of IRS-1, converting it into an inhibitor of IR tyrosine kinase *in vitro*, and attenuating IR signalling. This has also been observed in other cell types. The production of TNF α by adipose tissue could be a local regulator of fat size, and the overproduction of TNF α in adipocytes of obese animals could represent a form of "adipostat", i.e. a normal homeostatic mechanism designed to limit adipocyte size in the face of overconsumption (Spiegelman, 1993 ; Spiegelman & Hotamisligil, 1993).

At the onset of lactation, the rate of lipogenesis increases greatly in mammary epithelial cells (Vernon & Flint, 1983), while it decreases in adipose tissue (Vernon, 1988), in part due to reciprocal changes in the amount of the regulatory enzyme acetyl CoA carboxylase and its mRNA in adipose tissue (Kim & Tae, 1994). This facilitates the targeting of the precursors for fatty acid synthesis to the mammary gland to satisfy the increased metabolic demand created by the requirement to synthesise and secrete milk. Insulin increases both the gene transcription and therefore the amount and the activation status of the enzyme (refer to chapter 1, section 1.5), while catecholamines decrease the activation status. Lactation alters the ability of adipocytes to respond to insulin, catecholamines and adenosine (Vernon, 1988). *In vitro* studies with adipose tissue from rats and sheep showed that insulin stimulation of lipogenesis was decreased during lactation (Burnol *et al.*, 1986; Vernon & Finley, 1988), although not all aspects of insulin action are impaired. There was a small increase in stimulation of lipogenesis in the adipose tissue from lactating sheep when insulin was present for 24 hours, although the stimulation was less than that observed in tissue from non-lactating sheep, as seen in previous studies (Burnol *et al.*, 1986; Vernon & Finley, 1988). However, after a prolonged exposure to insulin and dexamethasone in subcutaneous adipose tissue from lactating sheep, there was a great increase in stimulation of lipogenesis, consistent with previous results (Vernon & Finley, 1988). $\text{TNF}\alpha$ reduced this increase in stimulated lipogenesis after 48 hours by half. $\text{TNF}\alpha$ inhibits many enzymes involved in fatty acid synthesis, including ACC synthesis (Pape & Kim, 1988) and lipoprotein lipase synthesis (Semb *et al.*, 1987), which is consistent with the observations seen here. However, the effect of $\text{TNF}\alpha$ is much smaller than that found with GH, which completely inhibits the increase in lipogenesis by insulin and dexamethasone in adipose tissue from lactating sheep (Vernon & Finley, 1988).

Thus, $\text{TNF}\alpha$ does not appear to be the putative secreted protein involved in GH regulation of lipolysis and lipogenesis in adipose tissue. I found that the effects of $\text{TNF}\alpha$ on basal lipolysis in rats and sheep were similar to those found by Green, and it also had similar effects to GH on inhibition of insulin-stimulated lipogenesis, but $\text{TNF}\alpha$ did not mimic all the effects of GH on adipose tissue metabolism, and its effects were quantitatively less than those of GH.

CHAPTER FOUR

DEVELOPMENT OF THE LIPOLYTIC SYSTEM IN OVINE PRE-ADIPOCYTES IN VITRO

4.1 Introduction

Elucidating the role of growth hormone (GH) in animal growth and development has been the goal of endocrinologists since Evans & Long (1921) observed that injections of crude hypophysial extracts promoted the growth of rats. Purification of the GH molecule (Li & Evans, 1944) and the molecular cloning of the GH cDNA (Miller *et al.*, 1980) have permitted extensive investigations into the effects of GH on physiological processes.

It is well established that GH regulates lipid and glucose metabolism *in vivo* (Jeanrenaud, 1965; Schwartz & Eden, 1985; Cameron *et al.*, 1987; Evock *et al.*, 1988), with adipose being the key target tissue. However, the mechanism through which GH modulates lipid accumulation remains unclear. Attempts to identify critical biochemical events regulated by GH have involved a variety of experimental approaches, including tissue explant or isolated cell culture systems (Watt *et al.*, 1991; Vernon *et al.*, 1995; Doris *et al.*, 1998), whole animal studies (McCutcheon & Bauman, 1986; Peters, 1986; Sechen *et al.*, 1990; Doris *et al.*, 1996), and a combination of whole animal treatments with GH followed by *in vitro* explant cultures (Vernon *et al.*, 1987; Lanna *et al.*, 1992; Doris *et al.*, 1994; Doris *et al.*, 1996). Such studies have shown that GH decreases lipogenesis, both by decreasing expression of lipogenic genes and by antagonising enzyme activation by insulin, and also enhances lipolysis. Effects of GH on lipolysis are complex, but a major mechanism appears to involve the attenuation of the ability of antilipolytic factors such as adenosine and prostaglandins to inhibit the process (Chapter 1, section 1.3.3).

However, it is not yet fully understood how these effects of GH on adipocyte cells are mediated, although it appears that the intracellular signalling pathways involved differ from those mediating the 'commitment to differentiate' effects of the hormone (Chapter 1, section 1.3.4). The initial objective of this study was to determine the suitability of an ovine adipocyte cell culture system for investigating the molecular basis of the lipolytic effects of GH, in particular the inhibitory effects of GH on adenosine inhibition of lipolysis.

4.2 Experimental procedure

4.2.1 Preparation of ovine adipocytes

Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase, as described in section 2.2.2.1. The pre-adipocytes were cultured in proliferation medium (consisting of Medium 199 containing Earle's salts, L-glutamine and 25mM Hepes (pH 7.4) and supplemented with 2mM acetate (pH 7.3), antibiotics (Penicillin G, 60µg/ml ; streptomycin sulphate, 100µg/ml) and 20% Newborn Calf Serum) in 24 well plates until they were confluent, which takes approximately 7 days. The cells were then washed once with Hanks Balanced Salt Solution (HBSS) and the medium was changed to differentiation medium (Dulbecco's MEM / Ham's F12 , supplemented with penicillin (60µg/ml) and streptomycin (100µg/ml)). Initial studies included insulin (1.6µg/ml), the lipid supplement ex-cyte (1% v/v) and T₃ (2nM) in the differentiation medium. Later studies also included dexamethasone (10nM) and rosiglitazone (BRL 49653 ; 100nM), as indicated. Control cells were still cultured in the proliferation medium.

4.2.2 Lipolysis Assay

10 days after the induction of cell differentiation (unless stated otherwise), the cells were washed and the medium was changed to 1 ml of Ringer's Bicarbonate medium (+ 3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes, pH 7.4) ; cells were incubated for 3 hours at 37°C. This contained either no addition (basal), adenosine deaminase (ad ; 8µg/ml), ad + different concentrations of isoproterenol, as indicated (isop ; 10^{-12} M - 10^{-4} M), or ad + isop + PLA (100nM). After the 3 hour incubation period, 800µl of medium was removed and assayed for glycerol content, as described in section 2.2.3.2. The number of cells per well was determined using a haemocytometer.

4.2.3 Statistical Analysis

Data were analysed by Restricted Maximum Likelihood (REML ; Patterson & Thompson, 1971). Each value contributing to a mean came from a different sheep, with the number of sheep being indicated in each figure legend.

4.3 Results

4.3.1 Determination of experimental conditions required to achieve the optimal rate of lipolysis in primary ovine adipocytes

A time course study was carried out to investigate the optimal time to measure the rate of lipolysis after the induction of cell differentiation to adipocytes, as seen in Figure 4.1. This shows that the basal rate of lipolysis increased with time. At the early stages of differentiation, there was no isoproterenol-stimulation of lipolysis. However, as cell differentiation increased the response to isoproterenol developed,

Time course data for the development of lipolysis in ovine pre-adipocytes

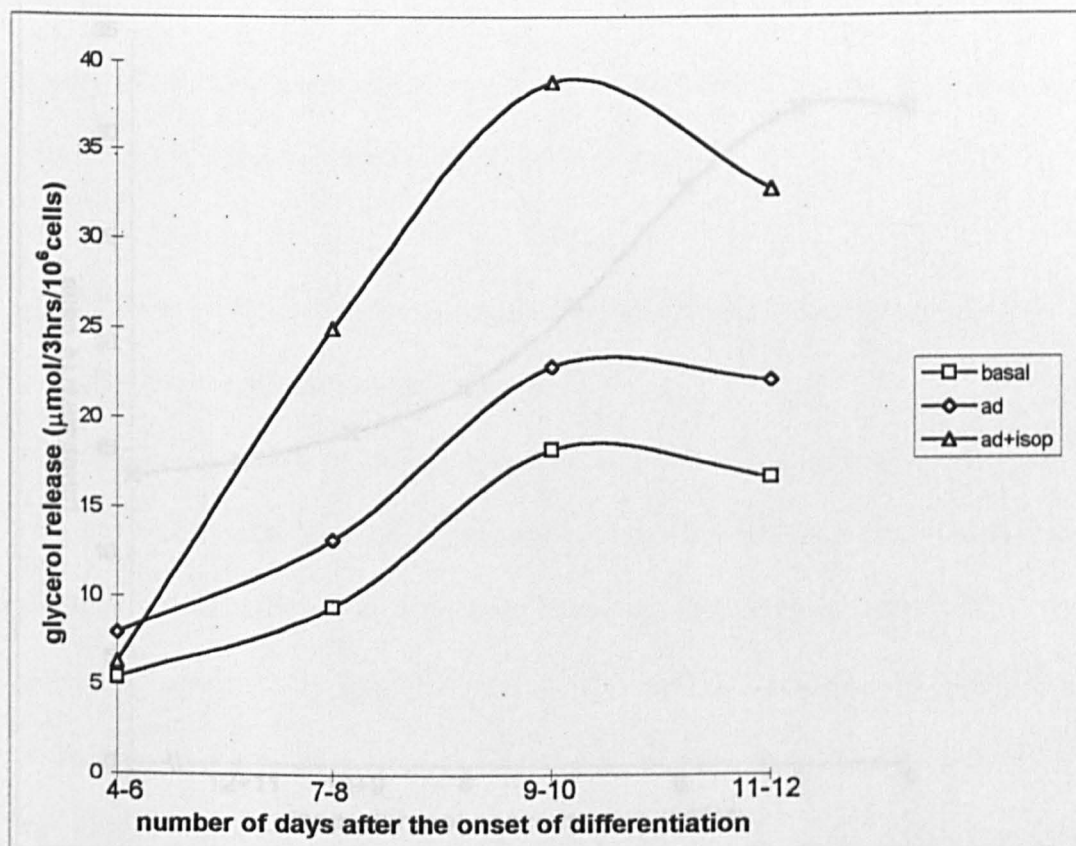


Figure 4.1. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium, which was supplemented with Ex-cyte, T₃ and insulin. Various days after the onset of adipocyte differentiation, the cells were washed and the medium changed to Ringer's Bicarbonate medium (3% BSA + 2mM sodium acetate + 1mg/ml D-glucose + 25mM Hepes, pH 7.4), with either no additions (basal), adenosine deaminase (ad) or adenosine deaminase + isoproterenol (10⁻⁵M) (ad+isop). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of between 5 and 11 observations.

Isoproterenol dose response data for lipolysis in ovine subcutaneous adipocytes

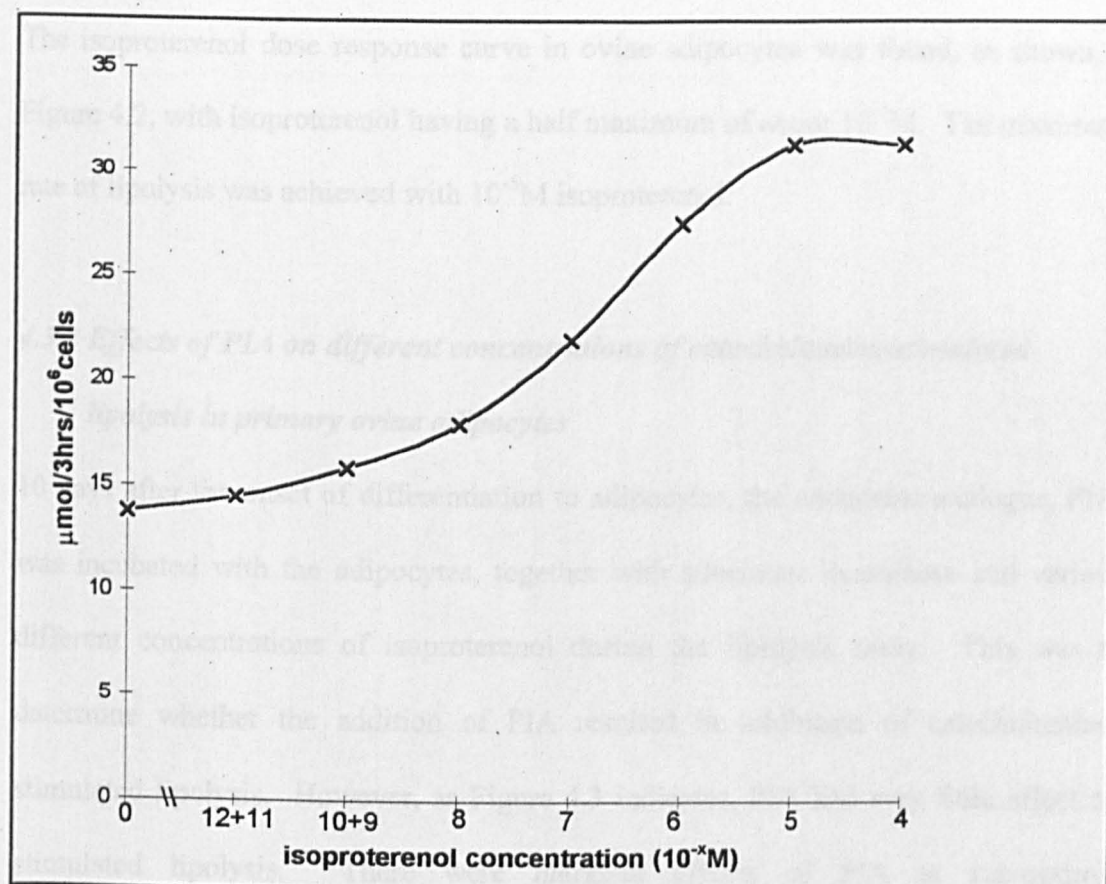


Figure 4.2. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate the pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium, which was supplemented with Ex-cyte, T_3 and insulin. 10 days after the onset of cell differentiation, the cells were washed and the medium changed to Ringer's Bicarbonate medium (+ 3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes, pH 7.4), containing adenosine deaminase and various concentrations of isoproterenol. After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of between 8 and 15 observations and were analysed using REML; effect of isoproterenol was significant, $P < 0.05$.

with the greatest isoproterenol stimulation of lipolysis being observed 10 days after the onset of differentiation.

The isoproterenol dose response curve in ovine adipocytes was found, as shown in Figure 4.2, with isoproterenol having a half maximum of about 10^{-7} M. The maximum rate of lipolysis was achieved with 10^{-5} M isoproterenol.

4.3.2 Effects of PLA on different concentrations of catecholamine-stimulated lipolysis in primary ovine adipocytes

10 days after the onset of differentiation to adipocytes, the adenosine analogue, PLA, was incubated with the adipocytes, together with adenosine deaminase and various different concentrations of isoproterenol during the lipolysis assay. This was to determine whether the addition of PLA resulted in inhibition of catecholamine-stimulated lipolysis. However, as Figure 4.3 indicates, PLA had very little effect on stimulated lipolysis. There were marginal effects of PLA at sub-optimal concentrations of isoproterenol (10^{-7} M- 10^{-6} M), but the effects were very small (much less than with ovine tissue explants, Chapter 3).

It has previously been observed that the presence of triiodothyronine (T_3) decreases response to adenosine in rats (Malbon *et al.*, 1988). However, differentiating cells in medium containing only insulin and ex-cyte did not increase the PLA inhibition of stimulated lipolysis (see Figure 4.4). T_3 had no significant effect on the rate of catecholamine-stimulated lipolysis itself in the presence of insulin and ex-cyte.

The effect of PIA (100nM) at different concentrations of
isoproterenol on lipolysis in ovine adipocytes

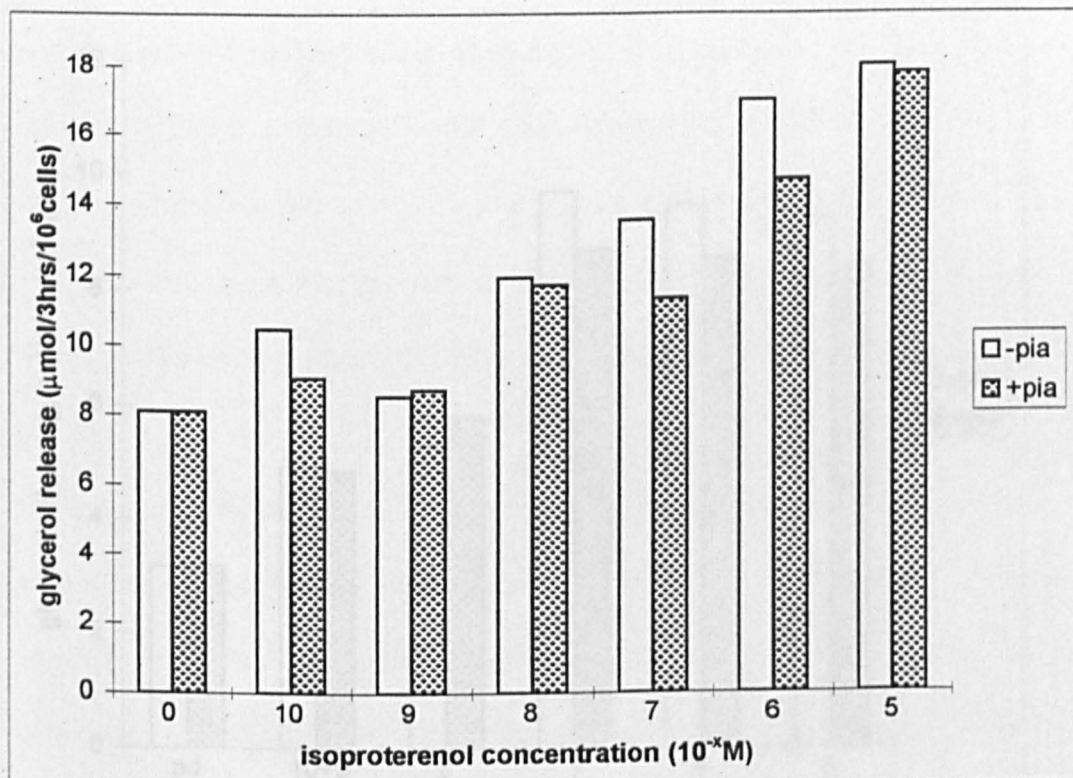


Figure 4.3. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate the pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium, which was typically supplemented with Ex-cyte, T_3 and insulin. 10 days after the onset of cell differentiation, the cells were washed and the medium changed to Ringer's Bicarbonate medium, containing adenosine deaminase, various different concentrations of isoproterenol, with or without the addition of 100nM PIA. After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of 4 or more observations, and were analysed using REML; PIA effect was significant, $P < 0.02$.

**The effect of PIA (100nM) at different concentrations of
isoproterenol on lipolysis in ovine adipocytes when cultured
without T₃**

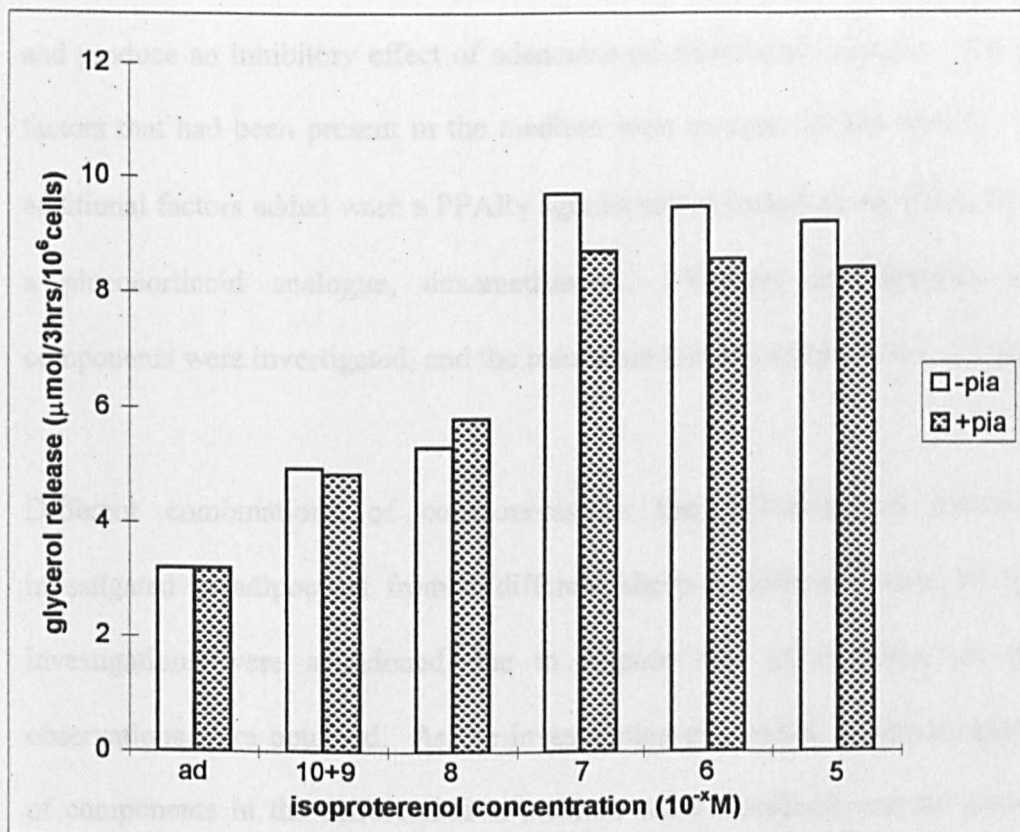


Figure 4.4. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate the pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium, which was supplemented with Ex-cyte and insulin, but no T₃. 10 days after the onset of cell differentiation, the cells were washed and the medium changed to Ringer's Bicarbonate medium (+ 3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes, pH 7.4), containing adenosine deaminase and various different concentrations of isoproterenol, with or without the addition of 100nM PIA. After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of 3 observations.

4.3.3 Altering the components of the differentiation medium for primary ovine

pre-adipocytes

The components present in the differentiation medium for the ovine pre-adipocytes were altered in an attempt to improve the rate of catecholamine-stimulated lipolysis and produce an inhibitory effect of adenosine on stimulated lipolysis. The previous factors that had been present in the medium were ex-cyte, insulin and T_3 . The two additional factors added were a PPAR γ agonist called rosiglitazone (BRL 49653) and a glucocorticoid analogue, dexamethasone. Various combinations of these components were investigated, and the results are shown in Figures 4.5, 4.6 and 4.7.

Different combinations of components in the differentiation medium were investigated in adipocytes from 6 different sheep. However, some of the initial investigations were abandoned due to a poor rate of lipolysis, so only two observations were obtained. As the investigation proceeded, different combinations of components in the differentiation medium were examined, and so there was an incomplete set of data for the 6 different sheep. As there was an overlap in the experiments, REML (Restricted Maximum Likelihood ; Patterson & Thompson, 1971) was used to analyse the data, as this package gives predicted values for missing data. Using REML to analyse the data produced several observations.

Figure 4.5 shows all the means for unstimulated and stimulated lipolysis for individual combinations of components in the differentiation medium. There was no difference in the rate of lipolysis between basal levels and the presence of adenosine deaminase for any combination of additions. Similarly, there was no difference between isoproterenol-stimulated lipolysis in the presence or absence of PIA.

However, there was a very significant difference between unstimulated and isoproterenol-stimulated lipolysis for all combinations ($P < 0.001$). Looking at the five different components individually, addition of ex-cyte, insulin and rosiglitazone had significant, positive effects ($P < 0.0001$ in each case), while the presence of T_3 was inhibitory ($P < 0.001$). In the absence of insulin, it was observed that the majority of the cells did not survive.

The REML analysis also indicated that there were significant ($P < 0.01$) interactions between components in the differentiation medium on their effects in isoproterenol-stimulated lipolysis. In the presence of insulin, dexamethasone and ex-cyte act in synergy. Individually with insulin, the rates of lipolysis were low, but when they were present together, the rate of lipolysis increased 2-3 fold. This gave a similar rate to when rosiglitazone and insulin were present together. A synergistic effect of dexamethasone and ex-cyte was also found in the presence of rosiglitazone and insulin; this combination resulted in the greatest rate of isoproterenol-stimulated lipolysis. As shown in Figures 4.5 and 4.6, the addition of T_3 had an inhibitory effect on the maximum rate of lipolysis found with insulin, ex-cyte, dexamethasone and rosiglitazone, but the presence of T_3 did not have any significant effect with any of the other combinations tested (insulin plus either ex-cyte, dexamethasone or rosiglitazone) (i.e. when lipolysis is not at its highest rate).

Differentiation in the presence of insulin, ex-cyte, dexamethasone and rosiglitazone not only markedly increased the maximum rate of isoproterenol-stimulated, the adipocytes became much more sensitive to isoproterenol ($ED_{50} = 2 \times 10^{-9} M$) (ED_{50} was $10^{-7} M$ after culture with ex-cyte plus insulin plus T_3); the addition of T_3 had no effect

on the ED_{50} value of isoproterenol. However, no inhibitory effect of PIA was observed on stimulated lipolysis using any of the combinations of components tested, when low or high rates of lipolysis were measured (Figures 4.5, 4.7), or when sub-optimal concentrations of isoproterenol were present (Figure 4.7).

The effect on lipolysis of adding different components to the differentiation medium in ovine adipocytes

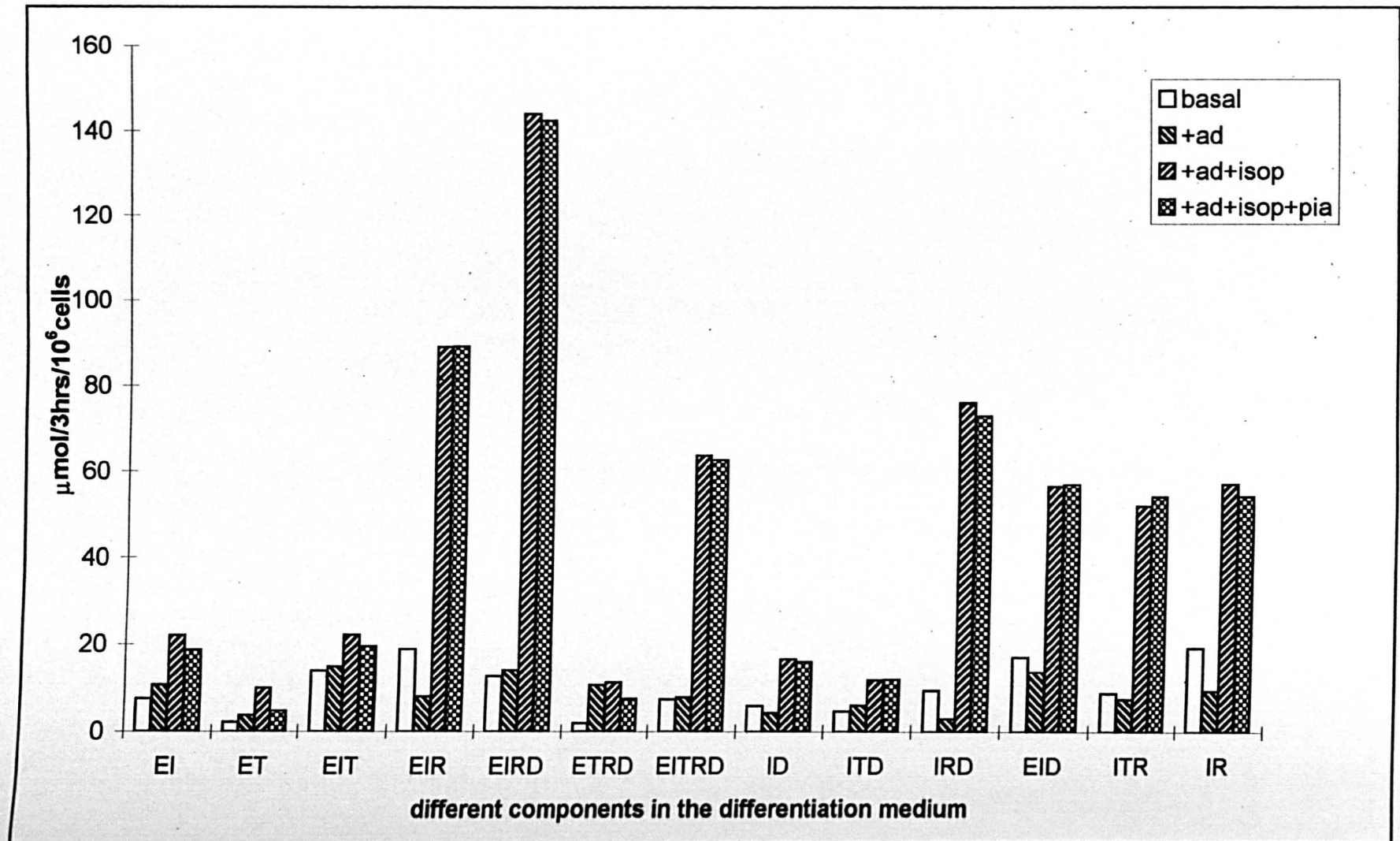
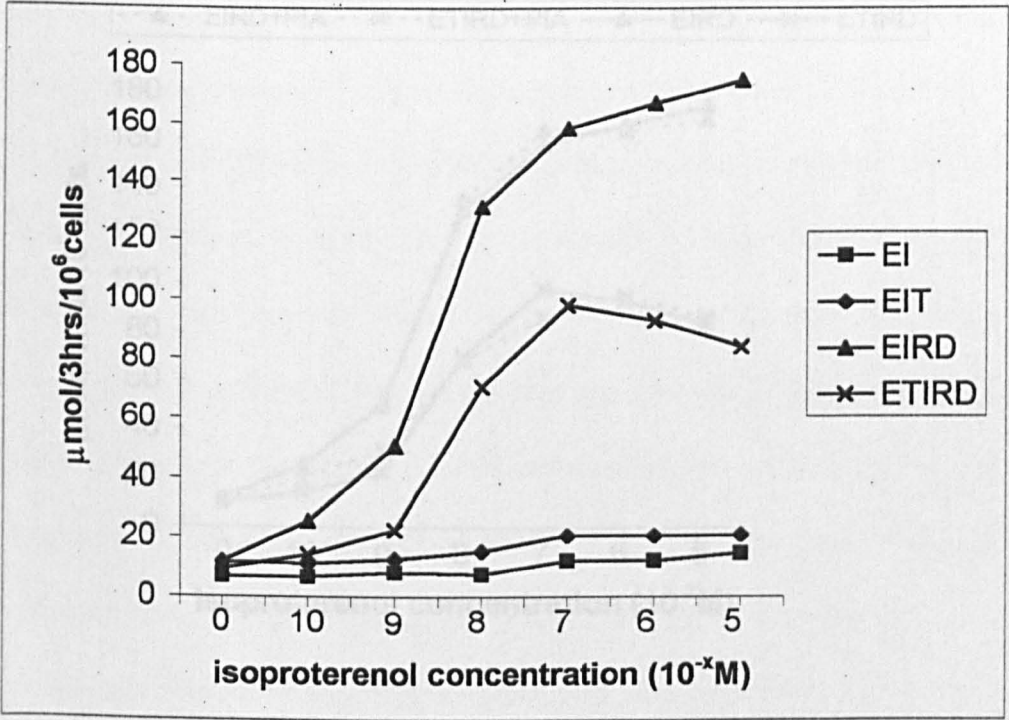


Figure 4.5. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate the pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium. The differentiation medium was supplemented with various combinations of insulin, T₃, ex-cyte, dexamethasone and rosiglitazone, as indicated. 10 days after the onset of cell differentiation, the cells were washed and the medium was changed to Ringer's Bicarbonate medium (+ 3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes, pH 7.4), containing either nothing (basal), adenosine deaminase (ad) or adenosine deaminase + isoproterenol (10⁻⁵M) (ad+isop). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of between 2 and 6 observations. Results were analysed using REML.

KEY : D = dexamethasone (10nM), E = ex-cyte (1%v/v), I = insulin (1.6µg/ml), R = rosiglitazone (100nM)
T = triiodo-L-thyronine (2nM)

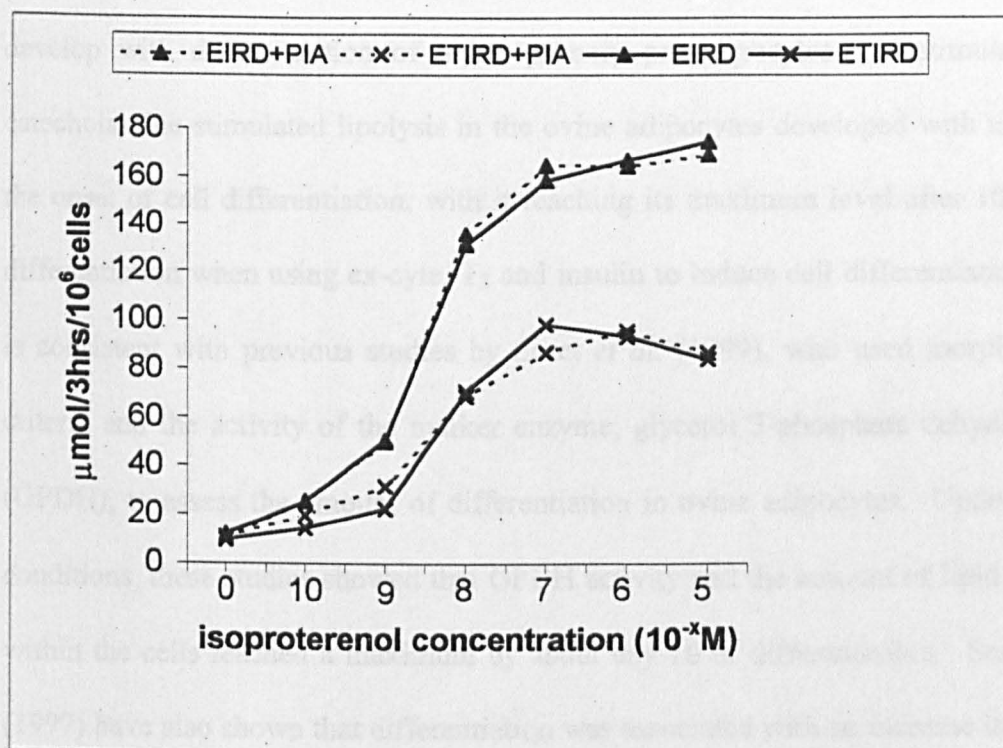
The effect of either omitting or including T_3 in the differentiation medium on lipolysis in ovine adipocytes



KEY : D = dexamethasone (10nM), E= ex-cyte (1%v/v),
 I = insulin (1.6 μ g/ml), R = rosiglitazone (100nM),
 T = triiodo-L-thyronine (2nM)

Figure 4.6. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate the pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium. The differentiation medium contained either ex-cyte+insulin +/- T_3 , or ex-cyte+insulin+dexamethasone+rosiglitazone+/- T_3 , as shown in the key. 10 days after the onset of cell differentiation, the cells were washed and the medium was changed to Ringer's Bicarbonate medium, (+3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes, pH 7.4), containing either nothing (basal), adenosine deaminase (ad) or ad+isoproterenol concentrations ($10^{-10}M$ - $10^{-4}M$). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of 3 observations for EI+/-T and 4 observations for EIDR+/-T.

The effect of PIA on lipolysis in ovine adipocytes in the presence and absence of T₃



KEY : D = dexamethasone (10nM), E= ex-cyte (1%v/v),
 I = insulin (1.6μg/ml), R = rosiglitazone (100nM),
 T = triiodo-L-thyronine (2nM)

Figure 4.7. Pre-adipocyte cell preparations were made from ovine subcutaneous adipose tissue by digestion with collagenase. The digest was then subjected to centrifugation to separate the pre-adipocytes from the adipocyte fraction. The pre-adipocytes were cultured in proliferation medium in 24 well plates until they reached confluence, then the medium was changed to differentiation medium. The differentiation medium contained ex-cyte+ insulin+dexamethasone+ rosiglitazone+/-T₃, as shown in the key. 10 days after the onset of cell differentiation, the cells were washed and the medium was changed to Ringer's Bicarbonate medium, (+3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes, pH 7.4), containing either adenosine deaminase (ad)+isoproterenol concentrations (isop ; 10⁻¹⁰M - 10⁻⁵M) or ad+isop concentrations+ PIA (100nM). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of 4 observations.

4.4 Discussion

Initial studies showed that some, but not all, of the features of the lipolytic system develop with differentiation of ovine primary pre-adipocytes. Unstimulated and catecholamine-stimulated lipolysis in the ovine adipocytes developed with time after the onset of cell differentiation, with it reaching its maximum level after 10 days of differentiation when using ex-cyte, T₃ and insulin to induce cell differentiation. This is consistent with previous studies by Soret *et al.* (1999), who used morphological criteria and the activity of the marker enzyme, glycerol 3-phosphate dehydrogenase (GPDH), to assess the amount of differentiation in ovine adipocytes. Under similar conditions, these studies showed that GPDH activity and the amount of lipid droplets within the cells reached a maximum by about day 10 of differentiation. Soret *et al.* (1997) have also shown that differentiation was associated with an increase in the rate of fatty acid synthesis (lipogenesis) from acetate which was paralleled by an increase in the activity of acetyl CoA carboxylase, both increasing to a maximum at around 10 days of differentiation. When sensitivity of the differentiated ovine adipocytes to isoproterenol was measured, the ED₅₀ value was 10⁻⁷M isoproterenol (100nM), which was less sensitive than had previously been measured in ovine subcutaneous tissue explants (ED₅₀=21nM) (Vernon *et al.*, 1995). However, the concentration of isoproterenol to produce the maximum rate of stimulated lipolysis was the same as observed previously in explants (10⁻⁵M).

Although the ovine pre-adipocytes were being induced to differentiate under these conditions, and the rate of catecholamine-stimulated lipolysis increased with time after differentiation, no significant inhibition of stimulated lipolysis was observed in the presence of the adenosine analogue, PIA, unlike the effects observed *in vivo* or in

explant tissue culture (Doris *et al.*, 1996, 1998). It is known that hyperthyroidism in rats results in a decreased response to adenosine which is, at least partly, due to a decrease in adenosine receptor number (Malbon *et al.*, 1988). However, when the ovine pre-adipocytes were differentiated in the presence of insulin and lipid only in the differentiation medium, there was still no significant inhibitory effect of PIA.

More recent studies have shown that the inclusion of rosiglitazone and dexamethasone, along with ex-cyte, T₃ and insulin enhances differentiation of ovine pre-adipocytes (Soret *et al.*, 1999). The stromal-vascular fraction of adipose tissue contains endothelial cells, mast cells and blood cells, in addition to adipocyte precursor cells at different stages of development (Flint & Vernon, 1993). These precursor cells can be induced to proliferate and differentiate *in vitro* in chemically defined media. For cell differentiation to proceed, the committed pre-adipocyte first enters a growth arrest phase. Expression of numerous genes undergoes chronological changes, mainly at the transcription level, and genes inhibitory to adipogenesis are repressed. The transcription factors CCAAT/enhancer binding protein alpha (C/EBP) and peroxisome proliferation-activated receptor gamma (PPAR γ), which is largely adipocyte-specific, have been shown to transactivate adipocyte-specific genes. PPAR γ is expressed at low, but detectable, levels in pre-adipocytes, and its expression rapidly increases after the onset of differentiation, with maximum levels of expression being attained in mature adipocytes (Brun *et al.*, 1996; Chawla & Lazar, 1994). It is activated by arachadonic acid metabolites and also thiazolidinedione drugs such as rosiglitazone (BRL49653) (Spiegelman, 1998). Recent studies show that rosiglitazone enhances differentiation of human (Adams *et al.*, 1997) and bovine (Ohyama *et al.*, 1998), as well as ovine (Soret *et al.*, 1999) pre-adipocytes. It has

previously been found that the presence of lipid stimulated differentiation of ovine pre-adipocytes (Broad & Ham, 1983) and of rat pre-adipocytes (Björntorp *et al.*, 1980). Dexamethasone is a glucocorticoid analogue, and has been found to enhance differentiation in ovine (Broad & Ham, 1983; Casteilla *et al.*, 1991; Vierck *et al.*, 1996; Soret *et al.*, 1999) and bovine (Aso *et al.*, 1995) pre-adipocytes.

In this study, different combinations of dexamethasone, ex-cyte, T₃, rosiglitazone and insulin were included in the differentiation medium for ovine pre-adipocytes to try and increase the activity of the lipolytic system and hopefully induce a response to adenosine (PIA). The combination of insulin, ex-cyte, rosiglitazone and dexamethasone resulted in a 10-fold or so increase in maximum lipolytic rate and a 50-fold increase in sensitivity to the β -adrenergic agonist isoproterenol compared with the initial combination tried (insulin, ex-cyte, T₃).

With respect to individual components, it was immediately apparent that the cells showed little differentiation in the absence of insulin, as had previously been observed (Aso *et al.*, 1995; Adams *et al.*, 1996; Soret *et al.*, 1999), and so insulin was included in all further studies. Rosiglitazone resulted in a large increase in the rate of lipolysis with every combination that was tested, and this effect appeared to reflect a general improvement of differentiation, due to it being a potent agonist of the transcription factor PPAR γ (Lehmann *et al.*, 1995; Krey *et al.*, 1997). Chronic exposure of differentiated 3T3-L1 cells to rosiglitazone had no effect on isoproterenol-stimulated lipolysis (Souza *et al.*, 1998). However, rosiglitazone had a much greater effect on lipolysis and also lipogenesis in ovine cells than was observed on GPDH activity (Table 4.1).

The presence of ex-cyte always resulted in an increase in the rate of lipolysis with all combinations tested (insulin + dexamethasone, insulin + rosiglitazone, and insulin + rosiglitazone + dexamethasone). The possibility that the effect of ex-cyte was due to providing fatty acid and hence ligand for PPAR γ appears to be excluded by it enhancing lipolysis in the presence of rosiglitazone.

It has also previously been shown that prolonged exposure to dexamethasone increases response and sensitivity to β -adrenergic agonists in both rats and sheep, at least in part by increasing the number of β -adrenergic receptors (Vernon, 1992). Dexamethasone caused an increase in the rate of lipolysis in the present study, but this was only apparent when ex-cyte was also present (insulin + ex-cyte, insulin + ex-cyte + rosiglitazone); dexamethasone had no effect when combined with insulin and rosiglitazone in the absence of ex-cyte. This suggests there is a synergy between ex-cyte and dexamethasone, but the molecular basis for this is unknown.

T₃ did not have an effect on development of the lipolytic system under most conditions investigated (ex-cyte + insulin, dexamethasone + insulin, insulin + rosiglitazone). However, T₃ resulted in a substantial decrease with lipolytic response to isoproterenol when insulin, ex-cyte, rosiglitazone and dexamethasone were also present in the differentiation medium. This effect of T₃ was unexpected, as hyperthyroidism increases whereas hypothyroidism decreases response to isoproterenol in rat adipose tissue, and hypothyroidism decreased the lipolytic response to catecholamines in neonatal lambs. Furthermore, T₃ causes over a 2-fold

increase in β 3-adrenoceptor concentration in differentiating 3T3-F442A adipocytes due to an increase in the half-life of β 3-adrenoceptor mRNA (El Hadri *et al.*, 1996).

However, despite improving the rate of pre-adipocyte differentiation, improving sensitivity of the adipocytes to isoproterenol, and greatly enhancing the rate of catecholamine-stimulated lipolysis in the ovine adipocytes, no significant effect of PIA was observed for any of the combinations of components present, or in the presence of sub-optimal concentrations of catecholamines. Hypothyroidism in rats increases response to adenosine and prostaglandins (Malbon *et al.*, 1988; Vernon *et al.*, 1991), while chronic incubation of sheep adipose tissue with dexamethasone increased response to adenosine (PIA) in sheep adipose tissue (Vernon *et al.*, 1991). Nevertheless, neither the addition of dexamethasone nor removal of T_3 from the medium induced a response to PIA in the present system. This suggests that the adenosine A_1 receptors are either not present or not functional in the differentiated ovine adipocytes. It has been shown that in the clonal cell line Ob1771, the adenosine stimulatory (with respect to adenylyl cyclase) A_2 receptors are present in higher quantities in pre-adipocytes, and the inhibitory A_1 receptors are present in higher quantities in the mature adipocyte, but only from day 16 after the induction of cell differentiation (Børghlum *et al.*, 1996). These studies also showed that PIA had an inhibitory effect on isoproterenol-stimulated lipolysis 16 days after differentiation (Børghlum *et al.*, 1996). Ravid & Lowenstein (1988) found that 3T3-F442A pre-adipocytes appeared to express only stimulatory (A_2) adenosine receptors, and that inhibitory (A_1) adenosine receptors were only expressed after the onset of differentiation. However, an effect of PIA on cyclic AMP activity was only measured

at later stages of differentiation (from day 11 onwards) (Ravid & Lowenstein, 1988). Elks *et al.* (1987) also found that PIA had an inhibitory effect on isoproterenol-stimulated lipolysis in the differentiated 3T3-L1 cell line, although lipolysis was not measured prior to 9 days after differentiation. A response to PIA was found on lipolysis in hamster adipose adipocytes 8 days after differentiation (Saulnier-Blache *et al.*, 1991). However, Mersmann *et al.* (1997) have found that no inhibitory adenosine receptors are present in suckling pigs at early stages of development, and so they seem to play no role in the modulation of adipocyte lipid metabolism in the young pig. Therefore, it seems that the inhibitory adenosine A₁ receptors are not present in very early stages of adipocyte differentiation, and that the time of their expression in the differentiating adipocytes depends on species, as response to adenosine develops at different intervals depending on the cell culture system. A few studies were undertaken to measure response to adenosine in the ovine primary adipocytes at later stages of differentiation (day 15), but no effect of PIA was observed. However, the cells had been induced to differentiate under sub-optimum conditions (ex-cyte + insulin + T₃).

**Comparison between GPDH activity and lipolytic rate in the
presence of different additions to the differentiation medium of
primary ovine subcutaneous pre-adipocyte cells**

additions to culture medium	GPDH (nmol/min/mg protein)	lipogenesis (nmol/3hrs/10⁶ cells)	glycerol release (μmol/3 hrs/10⁶ cells)
Ex-cyte	53	601	20
Rosiglitazone	66	1806	64
Dexamethasone	61	270	16

Table 4.1. A comparison of the effects of dexamethasone, rosiglitazone and ex-cyte on GPDH activity and the rate of lipolysis in pre-adipocytes from suckling lambs and fattening sheep. Differentiation was induced by the addition of insulin (1.6μg/ml) and T₃ (2nM), with or without ex-cyte (1% v/v), dexamethasone (10nM) and rosiglitazone (100nM), as indicated. GPDH activity, rate of lipogenesis and lipolytic rate were assessed after 10 days of differentiation. Values for GPDH activity are taken from Soret *et al.* (1999) and values for the rate of lipogenesis are unpublished observations (Soret & Vernon).

CHAPTER FIVE

LIPOLYSIS AND LIPOGENESIS IN THE 3T3-F442A MURINE ADIPOCYTE CELL LINE

5.1 Introduction

The mechanisms whereby growth hormone (GH) and insulin regulate adipocyte metabolism have not yet been fully elucidated. It is well established that GH acts chronically to decrease adiposity in animals by several mechanisms, including a decrease in lipogenesis and an increase in lipolytic rates (Boyd & Bauman, 1989; Vernon & Flint, 1989; Goodman, 1993). It has previously been shown that GH acts chronically by itself to decrease the rate of lipogenesis and to antagonise insulin stimulation of lipogenesis in adipose tissue from various species (Nyberg & Smith, 1977; Vernon, 1982; Walton & Etherton, 1986; Etherton *et al.*, 1987). These adaptations are observed both *in vivo* and *in vitro* (Bauman & Vernon, 1993; Etherton *et al.*, 1993). In addition to its chronic insulin-antagonistic effects, GH also has an acute insulin-like effect in adipocytes (Vernon & Flint, 1989; Goodman, 1993), although the physiological significance of this is uncertain as it is only observed when adipocytes have not been exposed to GH for a period (Vernon & Flint, 1989; Goodman, 1993). GH also promotes differentiation of several pre-adipocyte cell lines, including 3T3-F442A cells (Green *et al.*, 1985).

The rate of lipogenesis is regulated by a number of enzymes. Acetyl CoA carboxylase (ACC) is the most important of these enzymes, and is subject to both chronic and acute control. It exists in both active and inactive states, with insulin and catecholamines causing an increase and decrease to the activation status of the enzyme, respectively, while insulin also acts chronically to increase gene transcription and therefore the amount of ACC (Vernon, 1992).

Several murine fibroblast cell lines (for example, 3T3-F442A and 3T3-L1) have the capacity to undergo differentiation into cells which exhibit most of the morphological (Green & Kehinde, 1976; Kuri-Harcuch & Wise, 1978) and biochemical (Kuri-Harcuch & Green, 1977; Wise & Green, 1978; Pairault & Green, 1979) characteristics of normal adipocytes. These cell lines were clonally isolated from Swiss 3T3 cells derived from disaggregated 17 to 19 day mouse embryos (Green & Meuth, 1974; Green & Kehinde, 1975; Green & Kehinde, 1976). Extensive studies using these pre-adipocyte cell lines indicate that they represent a reasonable model of the process of adipocyte differentiation *in vivo*. It has been shown previously that the 3T3-F442A adipocytes provide a versatile *in vitro* model for analysis of cellular events resulting in hormonal changes in lipogenic and lipolytic rates (Goodman & Schwarz, 1974; Schwarz, 1980; Schwarz *et al.*, 1985). As the ovine adipocytes did not appear to show any response to adenosine, a key target of the lipolytic action of GH (see chapter 4), the 3T3-F442A adipocyte cells were utilised to further elucidate the molecular actions of GH and also insulin on adipocyte metabolism.

5.2 Experimental procedure

5.2.1 Culture of 3T3-F442A pre-adipocytes

3T3-F442A pre-adipocytes were induced to proliferate in culture, as described in section 2.2.2.4. Two-day confluent pre-adipocytes were induced to differentiate, as described in section 2.2.2.5. 8 days after the onset of cell differentiation, unless stated otherwise, the insulin and FCS were removed from the medium.

5.2.2 *Lipolysis assay*

After an overnight period of serum and insulin removal, hormones were added to the medium for a 24 hour or a 48 hour period, unless stated otherwise. The rate of lipolysis was measured, as described in section 2.2.5.1.

5.2.3 *Lipogenesis assay*

After an overnight period of serum and insulin removal, insulin (100 ng/ml) and / or growth hormone (100 ng/ml) were added to the medium for a 24 hour or a 48 hour period prior to the measurement of the rate of lipogenesis. ^{14}C -acetate incorporation into adipocyte lipid was determined, as described in section 2.2.5.2. ^{14}C -glucose incorporation into adipocyte fatty acids was determined, as described in section 2.2.5.3. The rate of lipogenesis was expressed as $\mu\text{mol. acetate or glucose incorporated} / 4 \text{ hours} / \text{mg DNA}$. The amount of DNA present was determined, as described in section 2.2.5.4.

5.2.4 *Acetyl CoA carboxylase (ACC) assay*

Following the overnight period of insulin and serum removal from the 3T3-F442A adipocyte differentiation medium, and the chronic treatment of the adipocytes with hormones, as indicated in the results section 5.3, the proportion of active ACC was determined, as described in the figure legends and section 2.2.5.5, and the amount of ACC activity was expressed as $\text{picomol.} / \text{minute} / \text{mg DNA}$, as described in section 2.2.5.4.

5.2.5 Statistical analysis

Results are expressed as means \pm S.E.M., and the statistical analysis was by analysis of variance (ANOVA), unless stated otherwise.

5.3 Results

5.3.1 Characterisation of the development of the lipolytic system in the murine 3T3-F442A adipocyte cell line

3T3-F442A pre-adipocytes were induced to differentiate into mature adipocytes by the introduction of 10% Foetal Calf Serum (FCS) and insulin ($5\mu\text{g/ml}$) into the medium, after the cells had been maintained at confluence for 48 hours. After a further 8 days in the differentiation medium, the serum and insulin were removed. At various time intervals at, or after, the serum and insulin removal, the rate of catecholamine-stimulated lipolysis was assessed over a 3 hour period. This showed that the response of the adipocytes to isoproterenol developed over a 48 hour period after the removal of serum and insulin, as shown in Figure 5.1.

Figure 5.2 shows that the optimum rate of lipolysis was attained with an isoproterenol concentration of 10^{-6}M , with the half-maximum concentration being $2 \times 10^{-8}\text{M}$ isoproterenol. However, although the response of the 3T3-F442A adipocytes to catecholamine-stimulated lipolysis developed, no significant inhibitory effects of the adenosine analogue, PIA, were apparent on the stimulated lipolytic system, as shown in Figure 5.3.

Development of the lipolytic response to isoproterenol after the removal of serum and insulin from the differentiation medium

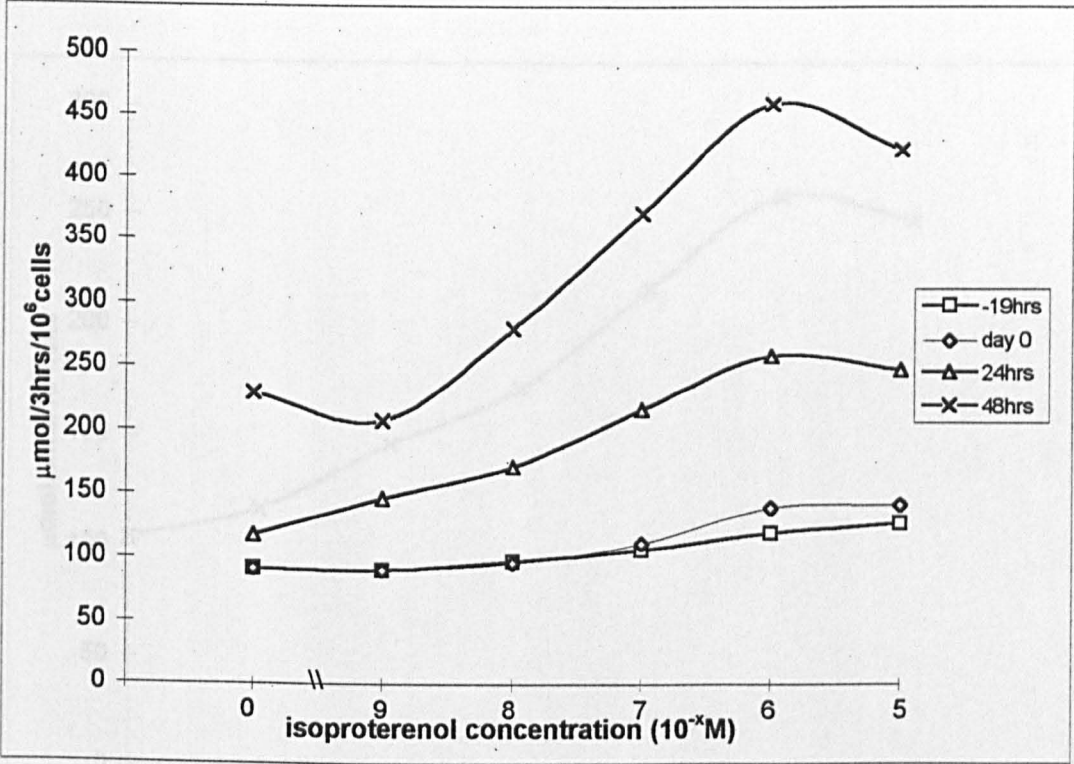


Figure 5.1. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium. At various intervals after the removal of serum, the cells were washed and the medium changed to Ringer's Bicarbonate medium (+3% BSA + 1mg.ml D-glucose + 2mM sodium acetate + 25mM Hepes), containing adenosine deaminase and various concentrations of isoproterenol. After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. The time intervals selected for the measurement of the rate of lipolysis were immediately after serum removal (-19hrs), the following day after serum removal (day 0), and 24 hours and 48 hours after the removal of the serum (24hrs, 48hrs, respectively). Results are the means of between 2 and 5 observations.

The effect of different concentrations of isoproterenol on the rate of lipolysis in 3T3-F442A adipocytes

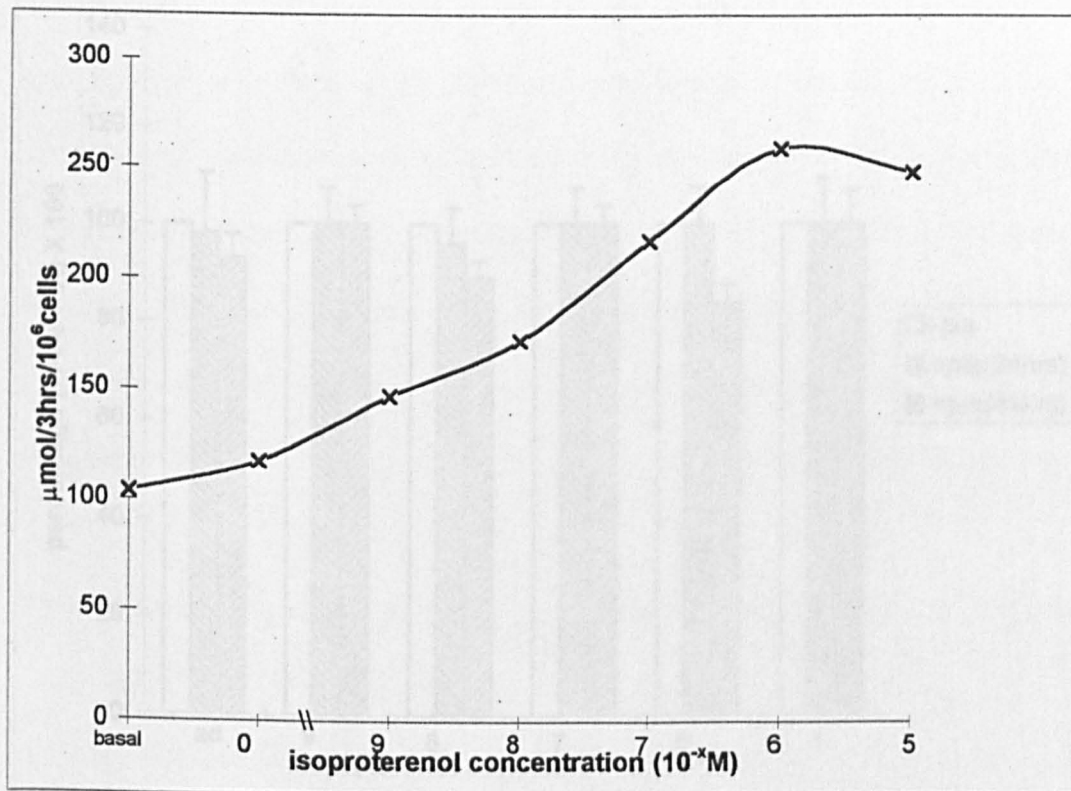


Figure 5.2. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin ($5\mu\text{g}/\text{ml}$), after which time the serum and insulin were removed from the medium. After the cells had been deprived of serum overnight plus an additional 24 hours, the cells were washed and the medium changed to Ringer's Bicarbonate medium (+3% BSA + $1\text{mg}/\text{ml}$ D-glucose + 2mM sodium acetate + 25mM Hepes), containing adenosine deaminase and various concentrations of isoproterenol. After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of 5 observations.

The effect of PIA (100nM) on different concentrations of isoproterenol on lipolysis in 3T3-F442A adipocytes

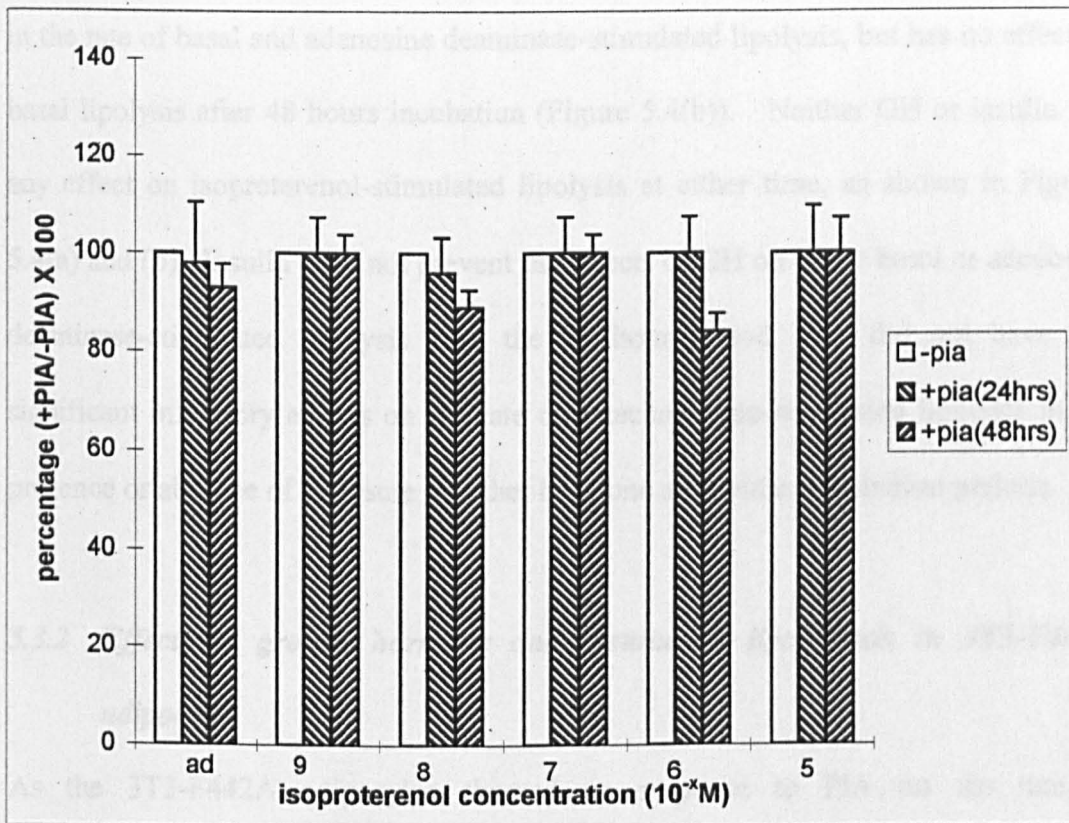


Figure 5.3. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5 μ g/ml), after which time the serum and insulin were removed from the medium. At various intervals after the removal of serum (24 hour or 48 hours after an overnight period with no serum), the cells were washed and the medium changed to Ringer's Bicarbonate medium (+ BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes), containing adenosine deaminase (ad), various concentrations of isoproterenol and plus or minus PIA (100nM). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measure of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of between 2 and 5 observations, + S.E.M.

The chronic effects of GH and insulin on the rate of stimulated and unstimulated lipolysis were investigated over a 24 hour and 48 hour incubation period. Figure 5.4(a) shows that the presence of GH for 24 hours results in a small increase ($P < 0.05$) in the rate of basal and adenosine deaminase-stimulated lipolysis, but has no effect on basal lipolysis after 48 hours incubation (Figure 5.4(b)). Neither GH or insulin had any effect on isoproterenol-stimulated lipolysis at either time, as shown in Figures 5.4(a) and (b). Insulin does not prevent the effects of GH on either basal or adenosine deaminase-stimulated lipolysis over the 24 hour period. PIA did not have any significant inhibitory effects on the rate of catecholamine-stimulated lipolysis in the presence or absence of exposure to either hormone after either incubation periods.

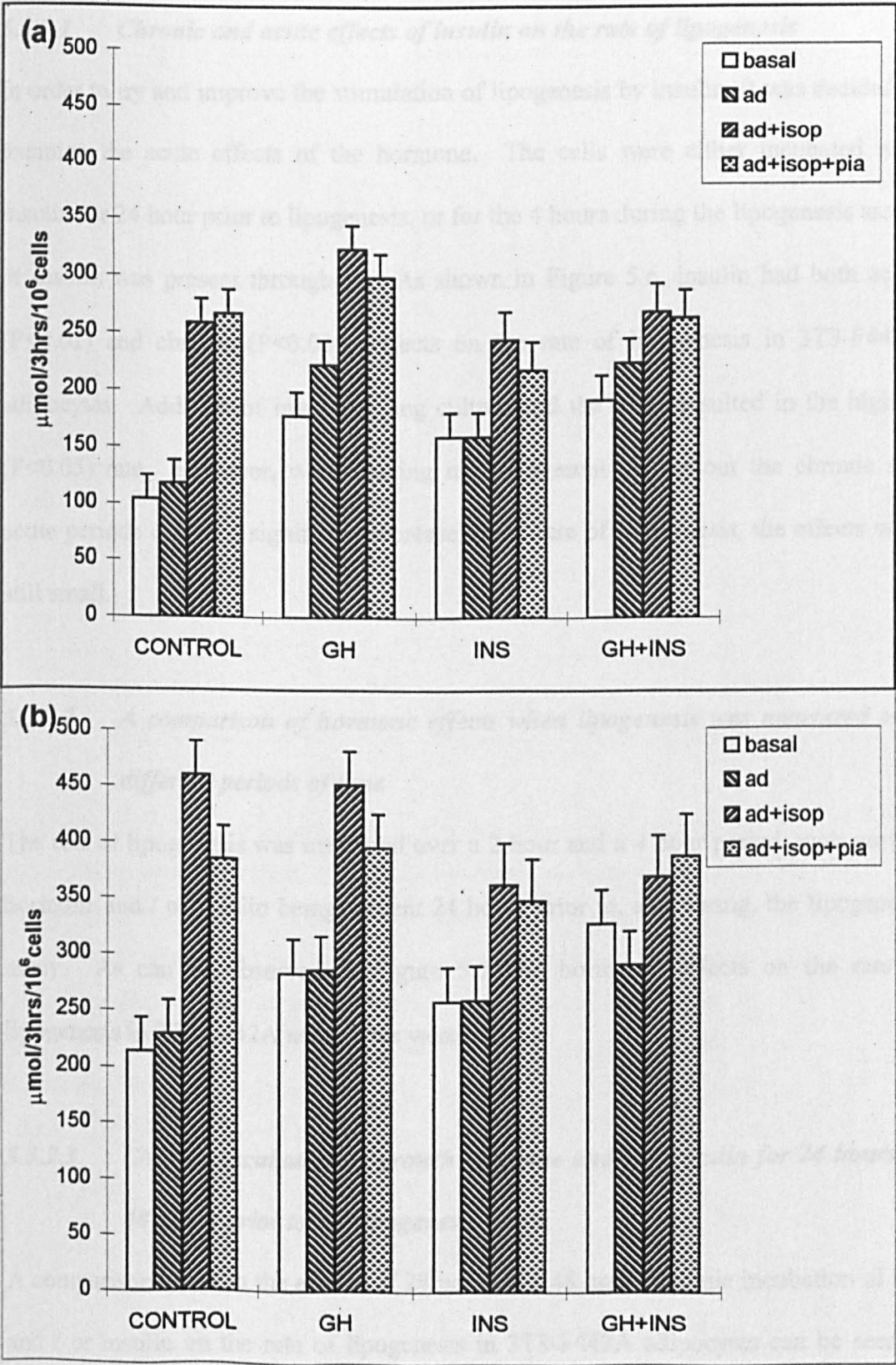
5.3.2 Effects of growth hormone and insulin on lipogenesis in 3T3-F442A adipocytes

As the 3T3-F442A adipocytes showed no response to PIA on the rate of catecholamine-stimulated lipolysis, it was decided to investigate the effects of GH and insulin on the lipogenic system instead, with a view to extending the previous observations by others in the laboratory on the roles of specific isoforms of protein kinase C on the modulation of lipogenesis by insulin and GH.

The rate of lipogenesis was assessed over a 4 hour period using ^{14}C -labelled sodium acetate incorporation into lipid as a marker. Eight days after the induction of 3T3-F442A differentiation followed by the removal of FCS and insulin for an overnight period, insulin and / or GH were added to the medium for a 24 hour incubation period. The hormones were removed from the medium immediately prior to the 4 hour period of the lipogenesis assay. Incubation with insulin resulted in an increase ($P < 0.05$) in

Figure 5.4. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5 μ g/ml), after which time the serum and insulin were removed from the medium. After the cells were serum-starved overnight, the hormones Growth Hormone (GH) and / or Insulin (INS) were added to the medium for either (a) 24 hours or (b) 48 hours prior to the cells being washed and the medium changed to Ringer's Bicarbonate medium (+ 3% BSA + 1mg/ml D-glucose + 2mM sodium acetate + 25mM Hepes). This medium contained either nothing (basal), adenosine deaminase (ad), isoproterenol (isop) (10^{-6} M) or PIA (100nM). After a 3 hour incubation period, medium was removed and assayed for glycerol content, which is a measurement of the rate of lipolysis. The number of cells per well was determined using a haemocytometer. Results are the means of 3 or 4 observations, + S.E.M.

The effect of Growth Hormone and Insulin on the rate of lipolysis
in 3T3-F442A adipocytes



lipogenesis, while incubation with GH reduced ($P<0.05$) the rate of lipogenesis, as shown in Figure 5.5. However, these hormonal effects were small.

5.3.2.1 *Chronic and acute effects of insulin on the rate of lipogenesis*

In order to try and improve the stimulation of lipogenesis by insulin, it was decided to examine the acute effects of the hormone. The cells were either incubated with insulin for 24 hour prior to lipogenesis, or for the 4 hours during the lipogenesis assay, or insulin was present throughout. As shown in Figure 5.6, insulin had both acute ($P<0.01$) and chronic ($P<0.001$) effects on the rate of lipogenesis in 3T3-F442A adipocytes. Addition of insulin during culture and the assay resulted in the highest ($P<0.05$) rate. However, while having insulin present throughout the chronic and acute periods caused a significant increase in the rate of lipogenesis, the effects were still small.

5.3.2.2 *A comparison of hormone effects when lipogenesis was measured over different periods of time*

The rate of lipogenesis was measured over a 2 hour and a 4 hour period, with growth hormone and / or insulin being present 24 hours prior to, and during, the lipogenesis assay. As can be observed in Figure 5.7, the hormonal effects on the rate of lipogenesis in 3T3-F442A adipocytes were linear.

5.3.2.3 *Chronic incubation of growth hormone and / or insulin for 24 hours or 48 hours prior to the lipogenesis assay*

A comparison between the effects of 24 hours and 48 hours chronic incubation of GH and / or insulin on the rate of lipogenesis in 3T3-F442A adipocytes can be seen in

**The effect of Growth Hormone and Insulin on the rate of
lipogenesis in 3T3-F442A adipocytes when present for 24 hours
prior to the measurement of lipogenesis**

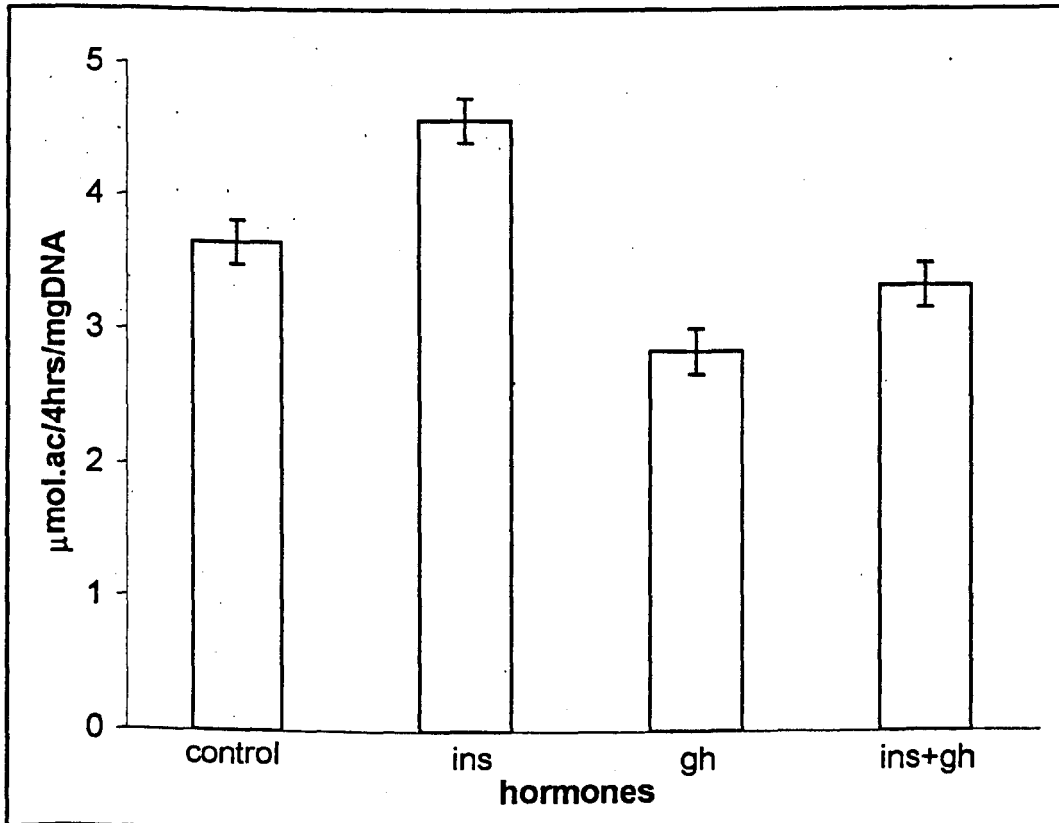


Figure 5.5. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin ($5\mu\text{g/ml}$), after which time the serum and insulin were removed from the medium. After the cells were serum-starved overnight, the hormones Growth Hormone (gh ; 100ng/ml) and / or Insulin (ins ; 100ng/ml) were added to the medium for 24 hours prior to, but not during, the measurement of the rate of lipogenesis. This was determined by measuring the amount of ^{14}C -labelled sodium acetate incorporated into the cell lipid over a 4 hour period. Results are the means of 16 observations, \pm S.E.M.

The chronic (24 hours) and acute (4 hours) effect of Insulin on the rate of lipogenesis in 3T3-F442A adipocytes

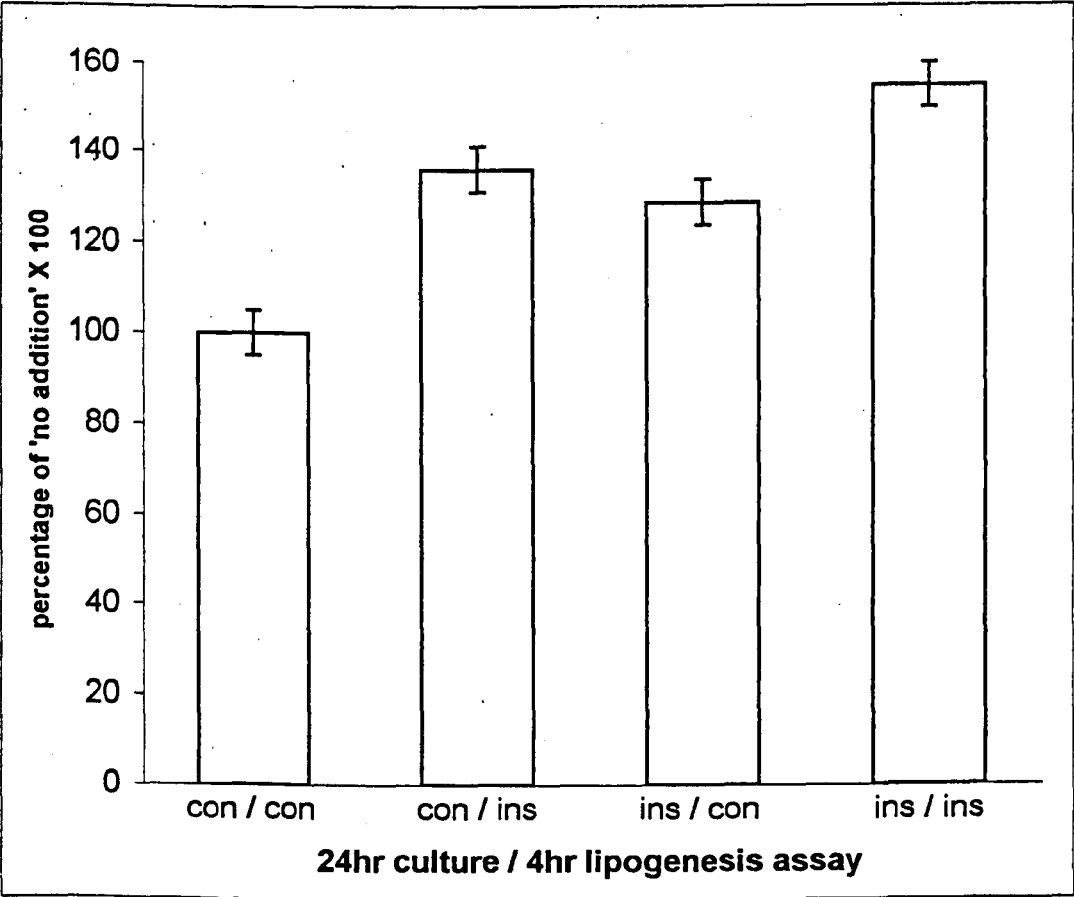


Figure 5.6. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum, after which time the serum was removed from the medium. After the cells had been serum-starved overnight, Insulin (ins ; 100ng/ml) was added to the medium for either 24 hours culture prior to, or 4 hours during, (or both) the measurement of the rate of lipogenesis (con = control). This was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into the cell lipid over a 4 hour period. Results are the means of 16 observations, +/- S.E.M.

The effect of Growth Hormone and Insulin on the rate of lipogenesis in 3T3-F442A adipocytes when present for 24 hours prior to, and either 2 hours or 4 hours during, the measurement of the rate of lipogenesis

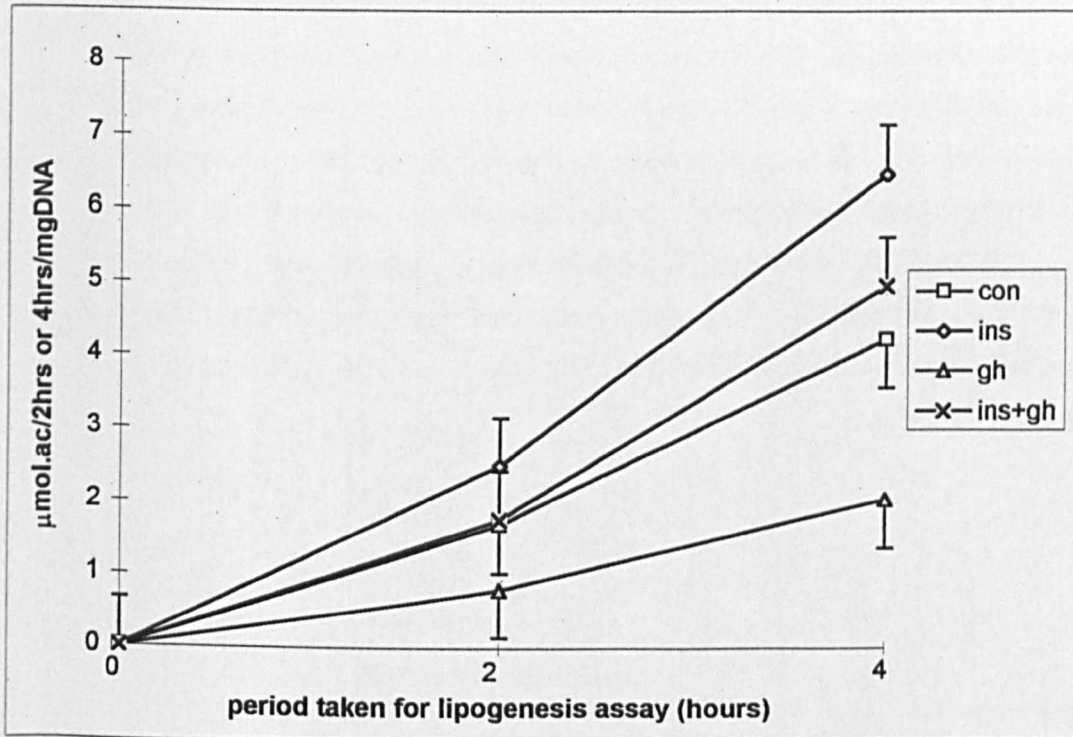


Figure 5.7. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium. After the cells had been serum-starved overnight, the hormones Growth Hormone (gh ; 100ng/ml) and / or Insulin (ins ; 100ng/ml) were added to the medium for 24 hours prior to, and during, the measurement of the rate of lipogenesis (con = control). This was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into the cell lipid over either a 2 hour period, or a 4 hour period. Results are the means of 5 observations, +/- S.E.M.

Figure 5.8. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5 μ g/ml), after which time the serum and insulin were removed from the medium. After the cells had been serum-starved overnight, the hormones Growth Hormone (gh ; 100ng/ml) and/or Insulin (ins ; 100ng/ml) were added to the medium for either 24 hours or 48 hours prior to, and during, the measurement of the rate of lipogenesis (con = control). This was determined by measuring the amount of 14 C-labelled sodium acetate incorporated into the cell lipid over a 4 hour period. (a) shows the absolute values for the rate of lipogenesis, and (b) shows the rates expressed as a percentage of 'no additions' X 100. Results are the means of 11 observations, +/- S.E.M.

The effect of Growth Hormone and Insulin on the rate of lipogenesis in 3T3-F442A adipocytes when present for either 24 hours or 48 hours prior to, and during, the measurement of the rate of lipogenesis

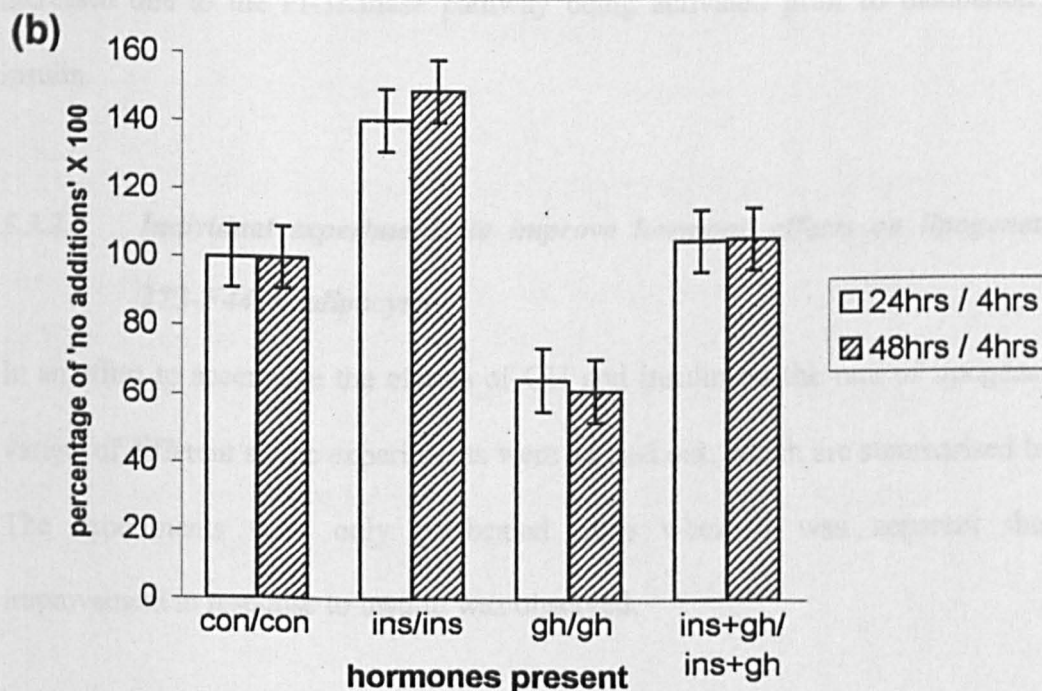
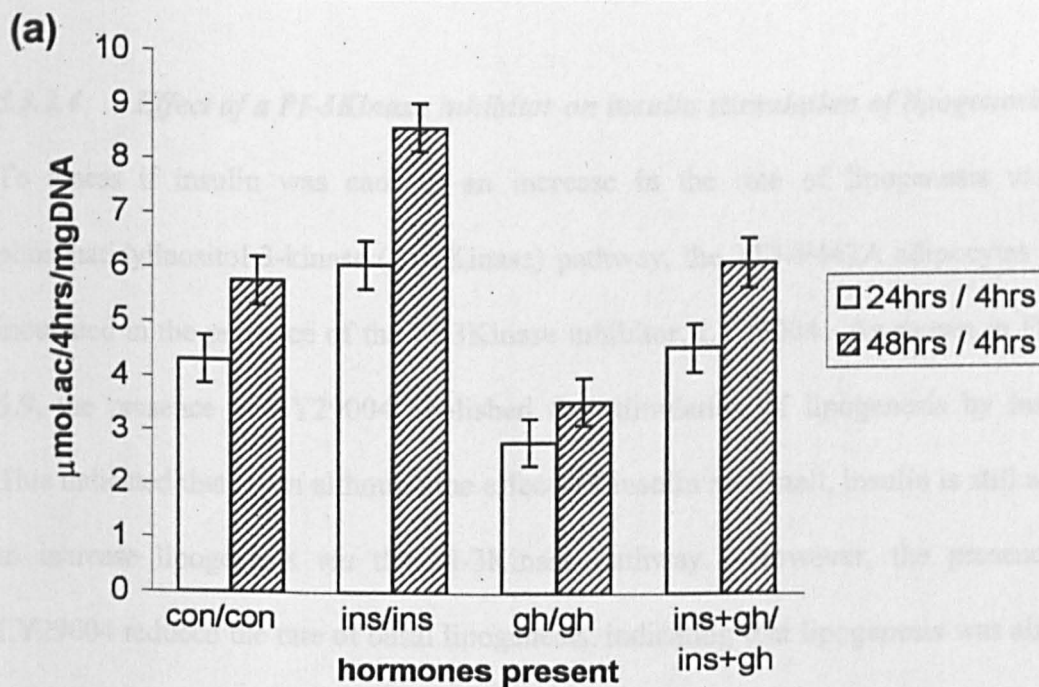


Figure 5.8, with the hormones being present during the 4 hour lipogenesis assay. As Figure 5.8(a) indicates, lipogenesis was greater after 48 hours incubation in the presence and absence of the hormones. However, the relative rate of lipogenesis did not increase or decrease in the presence of the hormones after the longer period of incubation, as shown in Figure 5.8(b).

5.3.2.4 *Effect of a PI-3Kinase inhibitor on insulin stimulation of lipogenesis*

To assess if insulin was causing an increase in the rate of lipogenesis *via* the phosphatidylinositol 3-kinase (PI-3Kinase) pathway, the 3T3-F442A adipocytes were incubated in the presence of the PI-3Kinase inhibitor, LY29004. As shown in Figure 5.9, the presence of LY29004 abolished the stimulation of lipogenesis by insulin. This indicated that, even although the effects of insulin are small, insulin is still acting to increase lipogenesis *via* the PI-3Kinase pathway. However, the presence of LY29004 reduced the rate of basal lipogenesis, indicating that lipogenesis was already increased due to the PI-3Kinase pathway being activated prior to incubation with insulin.

5.3.2.5 *Individual experiments to improve hormone effects on lipogenesis in 3T3-F442A adipocytes*

In an effort to accentuate the effects of GH and insulin on the rate of lipogenesis, a variety of different single experiments were carried out, which are summarised below. The experiments were only performed once when it was apparent that no improvement in response to insulin was observed.

The effect of the PI 3-Kinase inhibitor Ly29004 on the rate of lipogenesis in 3T3-F442A adipocytes in the presence and absence of insulin

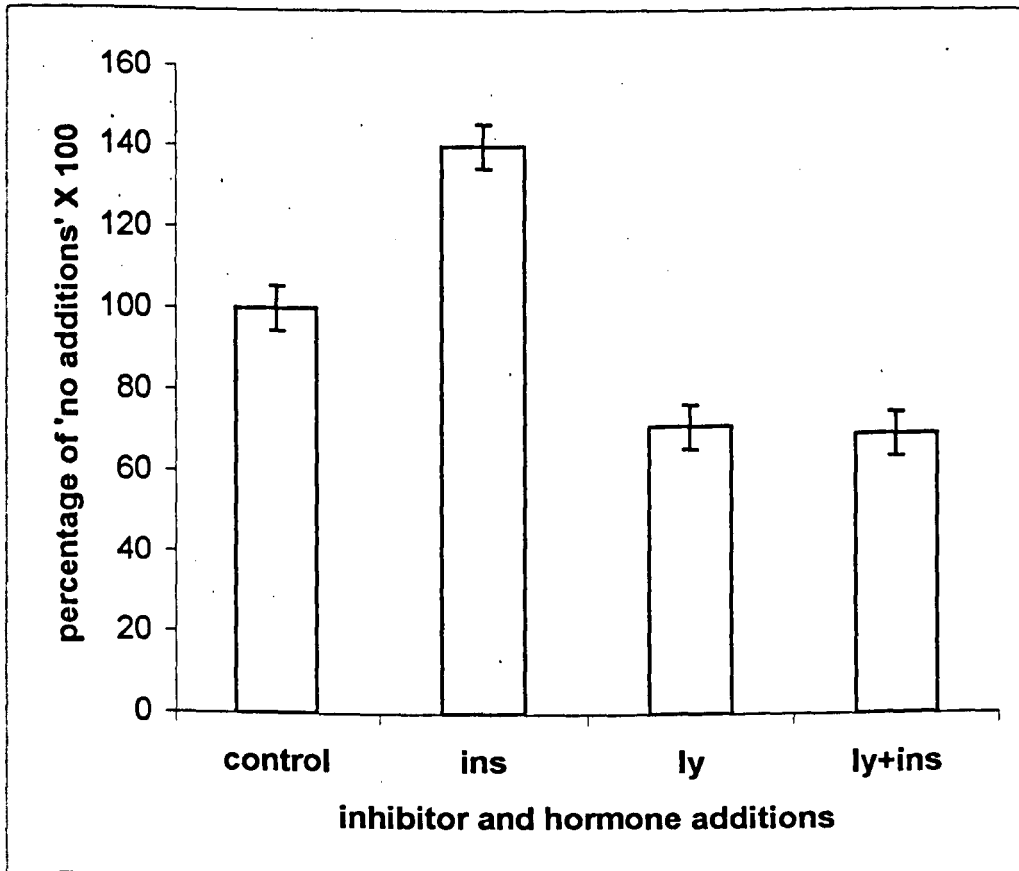


Figure 5.9. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5 μ g/ml), after which time the serum and insulin were removed from the medium. After the cells had been serum-starved overnight, Ly29004 (ly ; 50 μ M) and / or Insulin (ins ; 100ng/ml) were added to the medium for 24 hours culture prior to, and for 4 hours during, the measurement of the rate of lipogenesis. This was determined by measuring the amount of 14 C-labelled sodium acetate incorporated into the cell lipid over a 4 hour period. Results are the means of 4 observations, +/- S.E.M.

The rate of lipogenesis was assessed either 7 days or 10 days after the induction of differentiation, with the insulin and serum having been removed for an overnight period prior to 24 hour hormone incubation. This was to ensure that the hormones did not have a greater effect on lipogenesis at an earlier stage of cell differentiation. However, both insulin and GH had greater effects 10 days after the induction of adipocyte differentiation.

The presence of insulin and serum was initially required in the differentiation medium for the 3T3-F442A pre-adipocytes to be induced to differentiate. However, the typical insulin concentration present in the medium was high (5 μ g/ml), and this caused concern over whether this high concentration of insulin was resulting in the adipocytes becoming desensitised to the hormone over the chronic incubation period, thereby resulting in increased basal levels (and increased stimulation of PI-3Kinase) and a decrease in insulin stimulation of the rate of lipogenesis. The cells were differentiated in the presence of different concentrations of insulin, ranging from 0.1 μ g/ml to 5 μ g/ml. The highest concentration of insulin, which was the concentration used routinely in the medium, appeared to reduce the overall rate of lipogenesis. However, it did not cause a decrease in the chronic response of the adipocytes to insulin or GH.

It was also decided to investigate the effects of insulin and GH on the rate of lipogenesis at various time intervals after serum and insulin removal from the differentiation medium to ensure that the hormonal effects on the lipogenic system were not being repressed by the serum. The time after serum removal did not appear to alter the effects of insulin and GH on the rate of lipogenesis up to 72 hours. After a

period without serum the adipocytes enter apoptosis, so any effects that the absence of serum may have on lipogenesis were not investigated after a 72 hour period.

It had been questioned as to whether trace amounts of serum and insulin remaining in the wells after the medium had been changed could cause the effects of the hormones to be diminished, and therefore the PI-3Kinase signalling pathway being activated already. However, the number of time that the adipocytes were washed with PBS did not alter the hormone effects on lipogenesis either.

The 3T3-F442A pre-adipocytes were differentiated following a protocol by Fleming *et al.* (1998), which had 10% FCS and 5µg/ml insulin present (standard differentiation medium). Variations of this medium have been described by different research groups. Schwarz and colleagues routinely added the glucocorticoid analogue, dexamethasone, and the non-selective phosphodiesterase inhibitor, IBMX, in addition to antibiotics and insulin, to the differentiation medium for the first 48 hours after the induction of differentiation (modified differentiation medium) (Dietz & Schwarz, 1991). A comparison of the standard and modified differentiation medium showed that the hormone had a greater effect on the rate of lipogenesis when the cells were differentiated in the modified medium. However, the overall rate of lipogenesis in the presence or absence of hormones was greatly reduced when the cells were induced to differentiate in the modified medium. For example, basal lipogenesis was ten times higher in cells differentiated in standard rather than modified differentiation medium.

During the overnight period that serum and insulin were absent from the medium prior to the addition of GH and / or insulin, different components like heat-inactivated

FCS and the PPAR γ agonist, rosiglitazone, were added to the medium in an attempt to enhance the hormone effect on lipogenesis. However, none of these additions appeared to increase the effects of insulin and GH on the rate of lipogenesis in the 3T3-F442A adipocytes.

5.3.2.6 Effects of growth hormone and insulin on the rate of lipogenesis from glucose in 3T3-F442A adipocytes : a comparison with acetate

An investigation was undertaken comparing the rate of glucose incorporation into fatty acid with that of acetate, by measuring the rate of lipogenesis (fatty acid synthesis) in 3T3-F442A adipocytes using either ^{14}C -labelled glucose or ^{14}C -labelled sodium acetate. The hormones were added to the medium 24 hours prior to, and during, the lipogenesis assay. As shown in Figure 5.10, GH inhibited lipogenesis from acetate and glucose to a similar extent. However, insulin stimulated lipogenesis by a greater degree ($P < 0.05$) when the amount of glucose incorporation was measured compared with acetate incorporation.

5.3.2.7 Hormonal effects on the amount of acetyl CoA carboxylase (ACC) activity in 3T3-F442A adipocytes

The amount of total ACC activity and initial ACC activity was measured in the differentiated 3T3-F442A adipocytes after chronic exposure to GH and / or insulin, as shown in Figure 5.11. Insulin had no effect on total ACC activity. Incubation with GH resulted in a decrease ($P < 0.05$) in the amount of total ACC activity measured, but neither growth hormone nor insulin had any effect on the proportion of ACC in the active state.

5.3.2.8 Effects of growth hormone and insulin on ACC mRNA levels

The amount of ACC mRNA was measured after incubation of 3T3-F442A adipocytes for either 24 hours or 48 hours with or without insulin and, or, GH. The presence of insulin tended to increase the amount of ACC mRNA whereas GH had no apparent effect, as shown in Figure 5.12. ACC mRNA was measured by Dr. M.T. Travers using an RNase protection assay (Travers *et al.*, 1997).

5.3.2.9 Development of lipogenesis after serum removal

Further investigation into the acetate incorporation into 3T3-F442A adipocytes showed that the lipogenic system in these cells was already well switched on. After the overnight period following serum and insulin removal, the rate of lipogenesis did not fall, indicating that the system is very robust (see Figure 5.13). Indeed, the rate of basal lipogenesis did not change significantly over a further 48 hour period of culture in the absence of insulin.

A comparison between rates of lipogenesis from glucose and acetate in 3T3-F442A adipocytes : the differences in the rate of lipogenesis

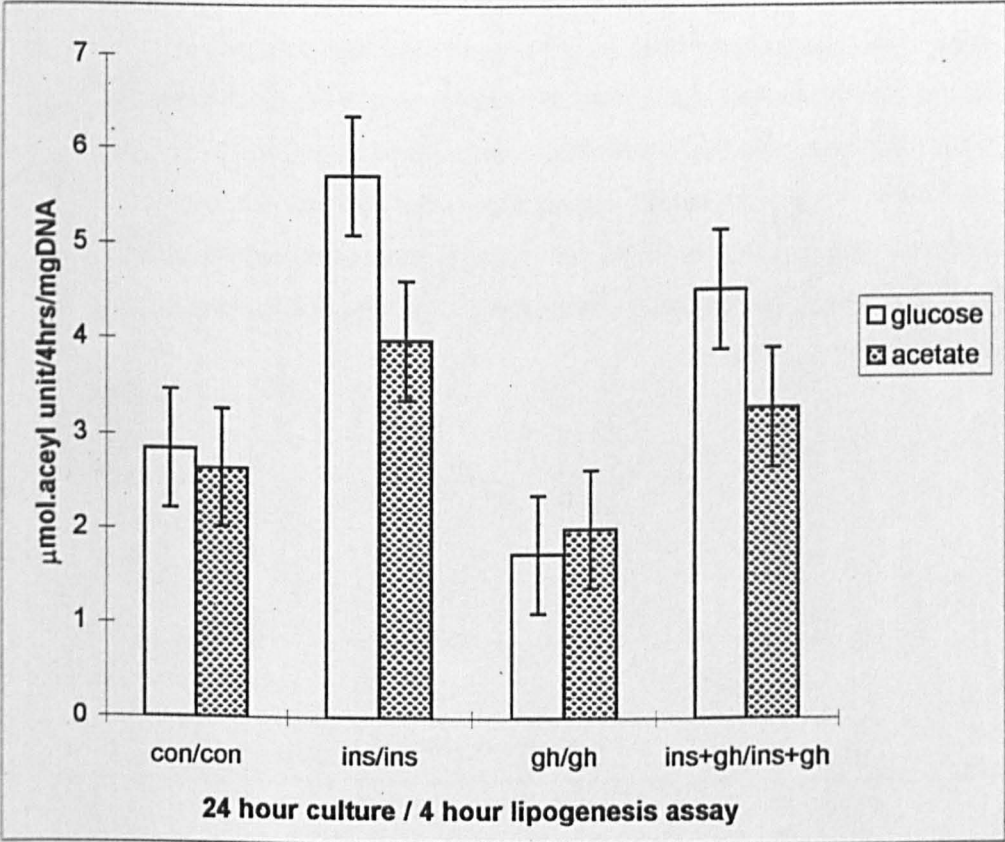
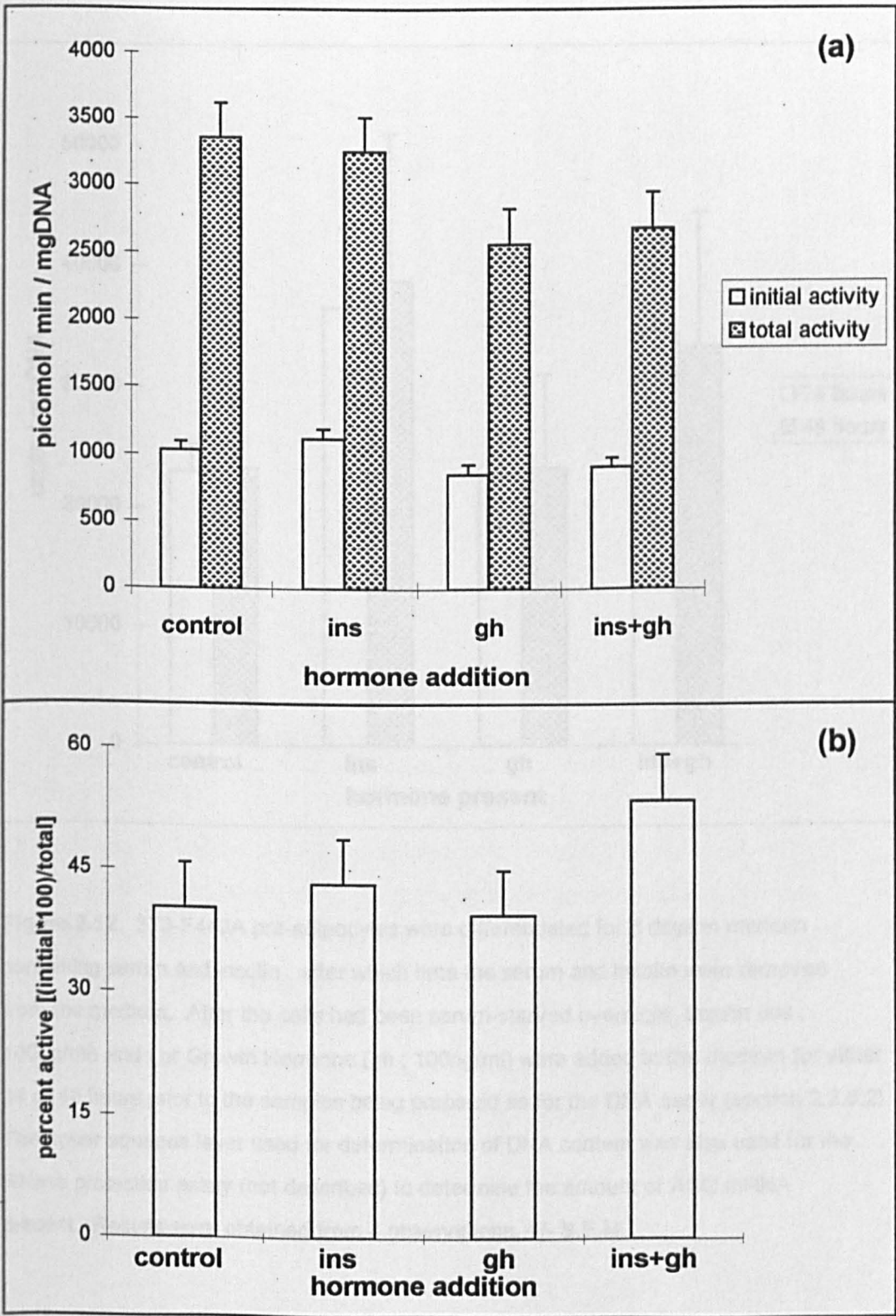


Figure 5.10. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium. After the cells were serum-starved overnight, the hormones Insulin (ins ; 100ng/ml) and / or Growth Hormone (gh : 100ng/ml) were added to the medium for 24 hours prior to, and the 4 hours during the measurement of the rate of lipogenesis (con = control). This was determined by measuring the amount of either ¹⁴C-labelled sodium acetate (0.5μCi/ml) or U-(¹⁻¹⁴C)-labelled D-glucose (0.1μCi/ml) that was incorporated into the cell lipid over a 4 hour period. Results are the means of 4 observations, +/- S.E.M.

Figure 5.11. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin , after which time the serum and insulin were removed from the medium. After the cells had been serum-starved overnight, Insulin (ins ; 100ng/ml) and / or Growth Hormone (gh ; 100ng/ml) were added to the medium for 24 hours prior to the ACC assay. The ACC activity was determined as outlined in section 2.2.5.5. Total ACC activity was the activity found after preincubation with citrate for 30 minutes (section 2.2.5.5). Initial activity, a measure of the activity of the enzyme in the active state, was measured as described in section 2.2.5.5. Results are means of 5 observations, + S.E.M.

Effects of Insulin and Growth Hormone on the amount of ACC

activity in 3T3-F442A adipocytes



Effects of Insulin and Growth Hormone on the amount of ACC

mRNA present in 3T3-F442A adipocytes

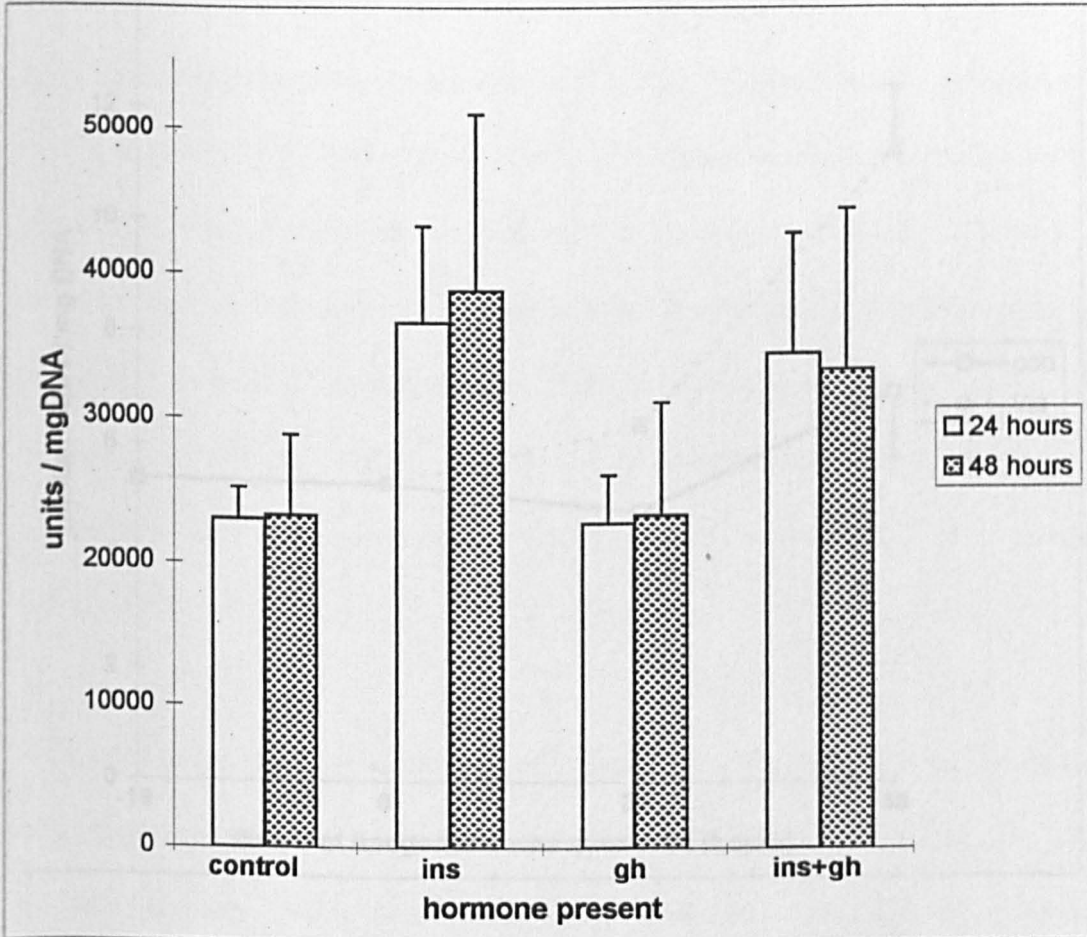


Figure 5.12. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin, after which time the serum and insulin were removed from the medium. After the cells had been serum-starved overnight, Insulin (ins; 100ng/ml) and / or Growth Hormone (gh; 100ng/ml) were added to the medium for either 24 or 48 hours prior to the samples being prepared as for the DNA assay (section 2.2.6.2). The upper aqueous layer used for determination of DNA content was also used for the RNase protection assay (not described) to determine the amount of ACC mRNA present. Results were obtained from 4 observations, \pm S.E.M.

Development of lipogenesis after the removal of serum and insulin from the differentiation medium in 3T3-F442A adipocytes

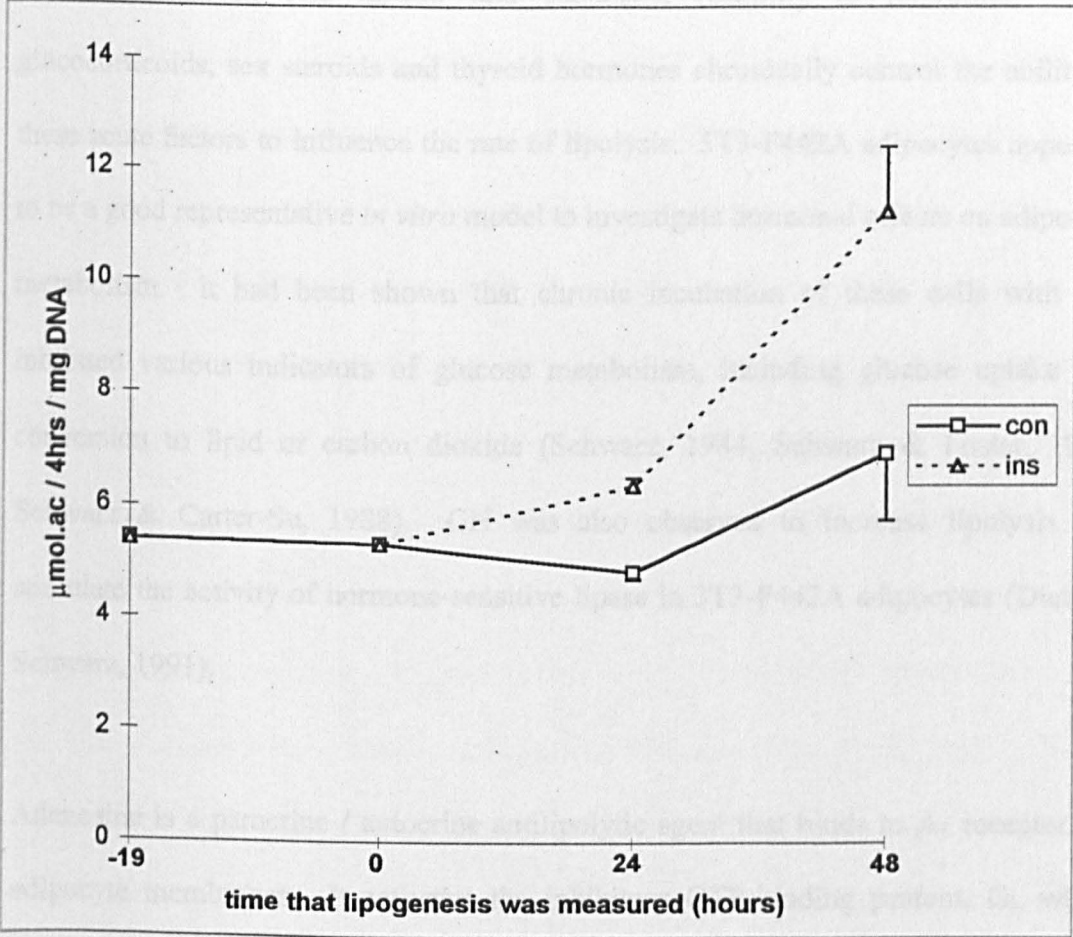


Figure 5.13. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium (-19 hours). After the cells were serum-starved overnight, insulin (ins ; 100ng/ml) was added to the medium for either 24 hours or 48 hours prior to, and during, the measurement of the rate of lipogenesis (con = control). This was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into the cell lipid over a 4 hour period. Results are the means of 3 observations, +/- S.E.M.

5.4 Discussion

Lipolysis is under complex acute control, with catecholamines and glucagon having a stimulatory effect, and insulin and adenosine resulting in inhibition. GH, glucocorticoids, sex steroids and thyroid hormones chronically control the ability of these acute factors to influence the rate of lipolysis. 3T3-F442A adipocytes appeared to be a good representative *in vitro* model to investigate hormonal effects on adipocyte metabolism. It had been shown that chronic incubation of these cells with GH inhibited various indicators of glucose metabolism, including glucose uptake and conversion to lipid or carbon dioxide (Schwarz, 1984; Schwarz & Foster, 1986; Schwarz & Carter-Su, 1988). GH was also observed to increase lipolysis and stimulate the activity of hormone-sensitive lipase in 3T3-F442A adipocytes (Dietz & Schwarz, 1991).

Adenosine is a paracrine / autocrine antilipolytic agent that binds to A_1 receptors of adipocyte membranes. It activates the inhibitory GTP-binding protein, G_i , which inhibits adenylate cyclase activity and hence lipolysis. Adenosine is released by adipocytes in amounts which inhibit cyclic AMP accumulation and lipolysis (Schwabe & Ebert, 1974; Schwabe *et al.*, 1973; Schwabe *et al.*, 1975). The external receptors that mediate inhibition (A_1) and stimulation (A_2) of adenylate cyclase possess different specificities towards adenosine analogues. For example, A_1 receptors show a greater affinity for the adenosine analogue PIA than the A_2 receptors (Londos & Wolff, 1977; Londos *et al.*, 1978; Londos *et al.*, 1980). It has been shown that GH can enhance lipolysis by decreasing the effects of agents acting *via* the G_i -based antilipolytic system, involving protein serine phosphorylation (Doris *et al.*, 1998).

The lipolytic system in 3T3-F442A adipocytes appeared to develop further after the removal of serum and insulin from the differentiation medium. This could possibly be due to the presence of a high concentration of insulin in the medium. Insulin elicits the phosphorylation of the β 2-adrenoceptor which could contribute to a decreased catecholamine responsiveness of adenylate cyclase (Haddock *et al.*, 1992). Engfeldt *et al.* (1988) have shown that exposure of human adipocytes to insulin leads to a translocation of β -adrenoceptors from the cell surface to an internal compartment. Chronic exposure to insulin also induces a decrease in β 3-adrenergic sensitivity due to β 3-adrenoceptor down-regulation (Fève *et al.*, 1994). There may also be other factors present in the serum which are responsible for a delay in lipolytic development.

The maximum response to isoproterenol was achieved with 10^{-6} M agonist (1 μ M), and the ED₅₀ (concentration giving a half-maximal effect) was 2×10^{-8} M (20 nM). This is the same as the concentrations of isoproterenol required to achieve the half-maximal response in ovine adipose tissue explants (Vernon *et al.*, 1995) and similar to that measured in differentiated ovine adipocytes (10^{-7} M) (refer to chapter 4). However, although a response to β -adrenoceptor agonist was observed in the 3T3-F442A adipocytes, no response to PIA (an adenosine analogue) was observed. No increase in the rate of lipolysis was observed after the addition of adenosine deaminase, which hydrolyses any exogenous adenosine present. These observations suggest that the inhibitory adenosine A₁ receptor was not present (or not functional) in these cells, although it has been shown previously that the cell lines 3T3-L1 and 3T3-F442A are responsive to adenosine after differentiation (Elks *et al.*, 1987; Ravid & Lowenstein, 1988) after approximately the same time period. Ravid & Lowenstein (1988) also

observed that, during the early phase of 3T3-F442A differentiation, the cells still retained their adenosine A₂ receptors, while new A₁ receptors were expressed. Two weeks after the induction of differentiation, the A₂ receptors had decreased substantially, but the A₁ receptors remained high (Ravid & Lowenstein, 1988). It remains to be investigated if the inhibitory adenosine A₁ receptor was present in the cells being utilised, or whether it appears later in the differentiation process (refer to chapter 4).

There was a small increase in both the basal and stimulated lipolytic rate in the presence of GH after 24 hours incubation. However, after 48 hours, GH failed to have a stimulatory effect, although it did reduce inhibition of lipolysis by insulin. This was also found by Glenn and colleagues (1992), who showed that GH did not affect glycerol release from 3T3-L1 adipocytes when exogenous insulin was absent from the cultures for over 48 hours, but that it did reverse insulin inhibition of lipolysis in 3T3-L1 adipocytes (Glenn *et al.*, 1992). Procedural differences (e.g. cell plating density for lipolysis assays, incubation times, medium supplements use of bGH rather than hGH) have been suggested for the discrepancy between present findings and the demonstration of GH-stimulated lipolysis in the absence of insulin with the 3T3-F442A adipocytes (Dietz & Schwarz, 1991).

The original aim of this study was to determine the mechanism of the effects of GH on the inhibition of lipolysis by adenosine. However, in view of the failure of the adenosine system to develop in either ovine adipocytes or 3T3-F442A adipocytes, this objective was abandoned. Concurrent studies in the laboratory concerned the mechanism of the effects of insulin and GH on the rate of lipogenesis in differentiated

3T3-F442A adipocytes, with particular emphasis on the involvement of different PKC isoforms in the signalling pathways (Millar, 1998). A new objective then was to determine the roles of PKC isozymes in the regulation of ACC expression and activity by insulin and GH.

Initial studies on the chronic effects of insulin and GH on the rate of lipogenesis in 3T3-F442A resulted in stimulation by insulin and a decrease in lipogenesis in the presence of GH when the hormones were present for 24 hours. GH also had an insulin-antagonistic effect on lipogenesis. However, these effects were small, and too small to investigate the role of specific PKC isoforms on lipogenesis. Insulin was found to have acute and chronic effects in the adipocytes, both resulting in stimulation of lipogenesis. There was an additive effect when insulin was present throughout chronic and acute incubation, but the increase was still small. When the hormones were present for a further 24 hours, the overall rate of lipogenesis increased. However, the percentage increase or decrease by insulin and GH, respectively, remained unaltered. Incubation with the known PI-3Kinase inhibitor, LY29004, confirmed that insulin was indeed having an effect on the lipogenic system *via* the PI-3Kinase signal cascade, and not by another pathway. Various different conditions for inducing differentiation in 3T3-F442A have been described (for example, Ravid & Lowenstein, 1988; Schwarz & Dietz, 1991; Plée-Gautier *et al.*, 1996; Raclot *et al.*, 1998), so different components in the medium were altered to try and enhance the insulin-stimulation of lipogenesis. However, there was no significant improvement. Insulin stimulation of lipogenesis from glucose incorporation was greater than that observed when measuring the amount of acetate incorporated into the adipocytes, but the effect was still small. This probably reflects the fact that the cells are from a

murine cell line, and there are certain basic differences between ruminants and non-ruminants with regard to fatty acid synthesis. It has been reported that the precursor for lipogenesis in adipose tissue is glucose in non-ruminants (Ballard *et al.*, 1969), whereas acetate rather than glucose has been shown to be the principle precursor in adipose tissue from ruminants such as sheep, cattle and goats (Ingle *et al.*, 1972; Whitehurst *et al.*, 1978; Liepa *et al.*, 1978).

The rate of lipogenesis is regulated by a number of enzymes, the most important of which is acetyl CoA carboxylase (ACC), and is subject to both chronic and acute control. ACC exists in both active and inactive states, with insulin and catecholamines causing an increase and decrease to the activation status of the enzyme, respectively, while insulin also acts chronically to increase gene transcription and therefore the amount of ACC (Vernon, 1992). GH decreases the amount of adipose tissue ACC and its mRNA (Vernon *et al.*, 1991 ; Liu *et al.*, 1994). However, when the amount of total and initial ACC activity was measured after chronic incubation with GH and insulin (and acute incubation), GH caused a slight reduction in both, but the percentage of ACC in the active state remained unaltered. No significant effect was detected in the presence of insulin. Insulin did tend to increase the amount of ACC mRNA per cell, but again the increase was not large enough to be confident in detecting any differences when looking at the effect of different PKC isoforms.

To ensure that the lack of an effect of insulin on the proportion of ACC in the active state was not an artefact due to a problem with the assay system, ovine subcutaneous adipose tissue was maintained in culture with and without 100ng/ml insulin for 24

hours, and the proportion of ACC in the active state determined. As found previously in ovine subcutaneous adipose tissue (Vernon *et al.*, 1996), insulin increased the proportion of the enzyme in the active state from 26.4% to 96.7%. Thus, the failure of insulin to activate ACC would not appear to be an artefact of the assay.

The reason for the very poor response to insulin is unclear. Curiously, maintaining the differentiated 3T3-F442A cells in culture in the absence of insulin (for up to at least 48 hours after the overnight serum deprivation) did not result in any fall in the rate of lipogenesis. This suggests that the lack of response to insulin may in part be due to lipogenesis being almost fully switched on during differentiation, and perhaps because of the absence of any agents to “switch” the process off in the system, there is little further scope for an effect of insulin.

To conclude, the 3T3-F442A fibroblasts developed many of the characteristics of adipocytes from primary culture with respect to the lipolytic and lipogenic systems. However, they did not appear to develop as well as had previously been reported. This could be due to the method of differentiation or some other undetected factor. As a result, the aims originally undertaken could not be investigated as there were not sufficient differences between basal and hormone-stimulated lipogenesis to confidently detect any reduction in hormone effect by further treatments.

CHAPTER SIX

STUDIES ON THE EFFECTS OF PHOSPHODIESTERASE (PDE) INHIBITORS ON LIPOGENESIS IN 3T3-F442A ADIPOCYTE CELLS

6.1 Introduction

The findings that the rate of lipogenesis did not change over at least 66 hours (overnight serum / insulin deprivation plus 48 hours) in the absence of insulin (Chapter 5) suggest that mechanisms which would normally operate to switch lipogenesis off in the absence of insulin were not functioning. For example, when sheep adipose tissue explants are incubated in the absence of insulin for several days, there is a progressive fall in the rate of lipogenesis which is prevented by insulin (Vernon & Sasaki, 1989). It thus seemed possible that reducing lipogenesis by the addition of isoproterenol might lead to a subsequent enhanced response to insulin. Lipolysis is stimulated acutely by catecholamines acting *via* the β -adrenergic receptor. This leads to activation of AC which catalyses the synthesis of cyclic AMP from ATP, thereby activating PKA and hormone-sensitive lipase (HSL) and enhancing the rate of lipolysis. Stimulation of lipolysis results in a concomitant reduction in lipogenesis. As shown in the present chapter, the addition of isoproterenol did indeed decrease the rate of lipogenesis in differentiated 3T3-F442A cells, with a concomitant enhanced response to insulin, but the effect of insulin was still small.

Insulin plays a key role in the regulation of lipid carbohydrate metabolism in many mammalian cells, principally liver, muscle and adipocytes. Despite recent substantial advances in the understanding of intracellular signalling, the detailed mechanisms by which insulin regulates these metabolic processes is still unclear. One important metabolic action of insulin is to block hydrolysis of stored triglycerides in adipocytes. The antilipolytic action of insulin can to a large extent be explained by the ability of this hormone to lower intracellular cyclic AMP levels, resulting in the reduction in the

activity of PKA, net dephosphorylation, and deactivation of HSL, and thereby inhibition of lipolysis and increased lipogenesis (Nilsson *et al.*, 1980; Beebe *et al.*, 1985; Londos *et al.*, 1985; Eriksson *et al.*, 1995). This effect of insulin is mainly mediated through phosphorylation and activation of phosphodiesterase (PDE) isoforms (see chapter 1.6). As the effect of insulin on lipogenesis was too small to warrant further investigation, the modulation of the effect on lipogenesis of isoproterenol by phosphodiesterases was explored.

6.2 Experimental Procedure

6.2.1 Culture of 3T3-F442A pre-adipocytes

3T3-F442A pre-adipocytes were cultured, as described in section 2.2.2.4. Two-day confluent pre-adipocytes were induced to differentiate, as described in section 2.2.2.5. 8 days after the onset of differentiation, the insulin and FCS were removed and the medium was replaced with low glucose medium (1.0 g/l).

6.2.2 Lipogenesis assay

Isoproterenol and / or PDE inhibitors were added to the medium for a 24 hour period after overnight serum and insulin starvation, unless stated otherwise. The concentrations of PDE inhibitors used were as follows ; rolipram (10 μ M), cilostamide (10 μ M), 8-methoxymethyl-IBMX (10 μ M) and IBMX (1mM), unless stated otherwise. Subsequently, the rate of lipogenesis was determined, as described in section 2.2.6.2.

6.2.3 Statistical analysis

Results are presented as means \pm S.E.M, and statistical analysis was by analysis of variance (ANOVA), unless stated otherwise.

6.3 Results

6.3.1 Incubation of isoproterenol with 3T3-F442A adipocytes – effect on lipogenesis

6.3.1.1 The effect of chronic and acute incubation with isoproterenol concentrations

Figure 6.1 shows the chronic effect on the rate of lipogenesis in 3T3-F442A adipocytes of increasing concentrations of isoproterenol. Concentrations of isoproterenol below 10^{-6} M had no effect on lipogenesis, but concentrations greater than 10^{-6} M resulted in the rate of lipogenesis decreasing as isoproterenol concentration increased.

Isoproterenol was included in the medium for different periods prior to the measurement of lipogenesis to determine if it had acute or chronic effects on lipogenesis in the 3T3-F442A adipocytes. The results shown in Figure 6.2 suggest that the effects of isoproterenol were both chronic and acute, as isoproterenol for a 24 hour period caused a greater reduction in the rate of lipogenesis than over 2 hours. The inclusion of isoproterenol during the 4 hour assay as well as during the 24 hour incubation period resulted in no greater maximum effect of isoproterenol on the rate of lipogenesis, but did move the dose response curve to the left such that inhibition was apparent with as little as 10^{-8} M isoproterenol. The rate of lipogenesis only partially recovered during the 4 hours of the assay after the isoproterenol had been removed. However, it is possible that trace amounts of isoproterenol could still have

The effect of different concentrations of isoproterenol on the basal rate of lipogenesis

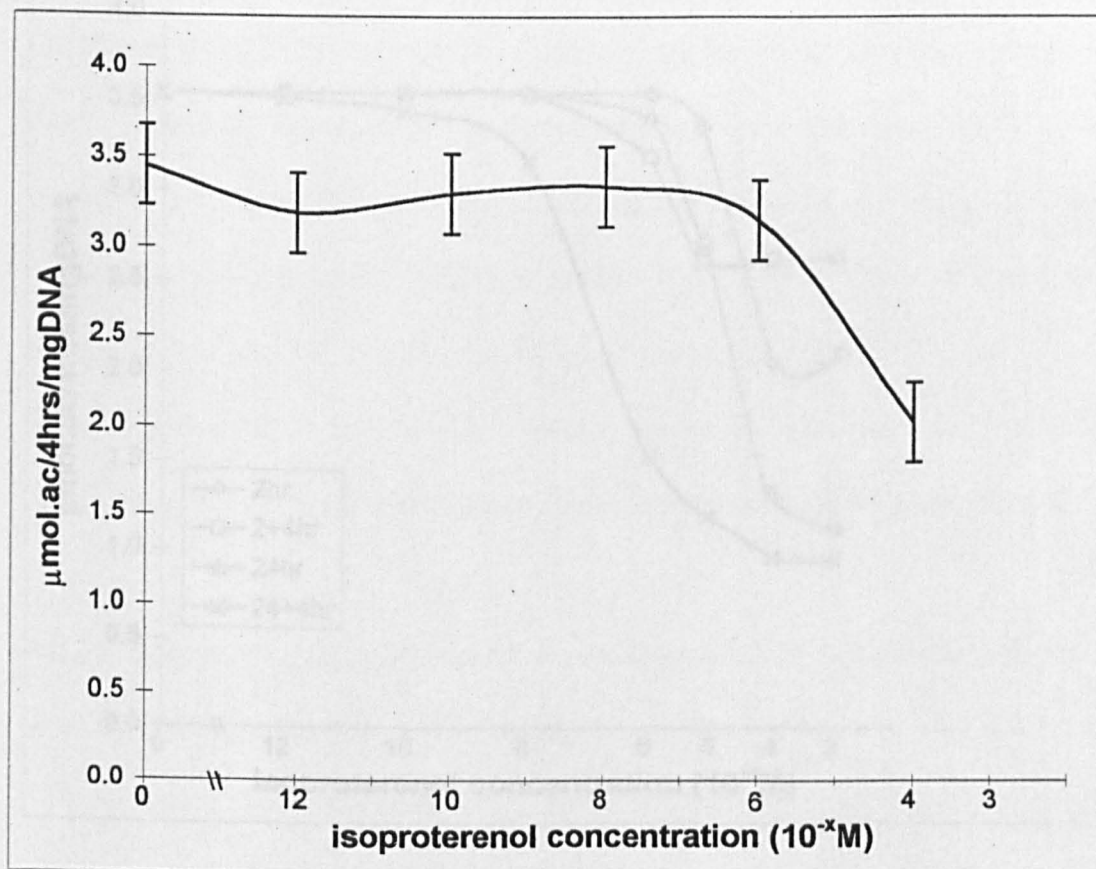


Figure 6.1. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium and various concentrations of isoproterenol were added for 24 hours prior to, but not during, the measurement of the rate of lipogenesis. This was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period. Results are the means of 8 observations +/- S.E.M.

**The effect of isoproterenol when it is present for various periods
prior to, or during, the measurement of the rate of lipogenesis**

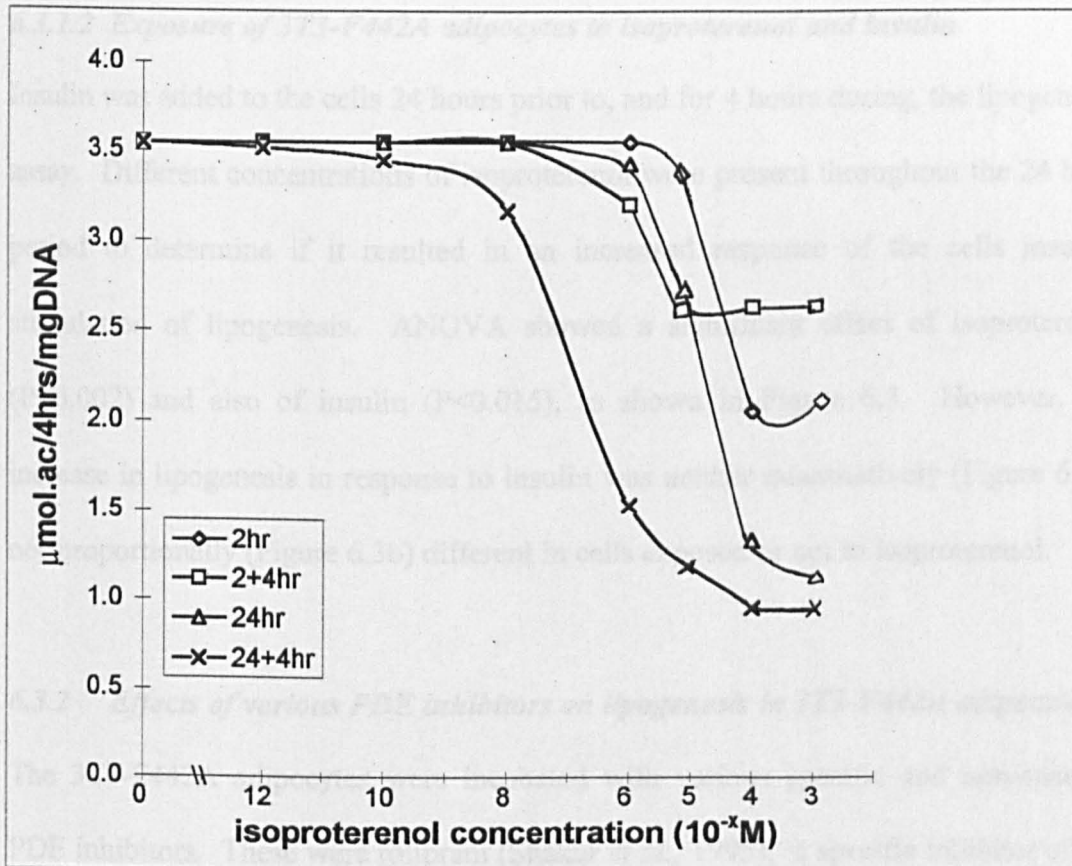


Figure 6.2. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium. Various concentrations of isoproterenol were added to the medium for either 24 hours or 2 hours prior to the measurement of the rate of lipogenesis. Isoproterenol concentrations were also either present or absent during the 4 hour period the ¹⁴C-labelled sodium acetate was incorporated into adipocyte lipid. Results are the means of 2 observations.

been present in the medium after the medium had been changed, despite the cells being washed between medium changes. This could account for the lack of recovery of the rate of lipogenesis after isoproterenol removal.

6.3.1.2 Exposure of 3T3-F442A adipocytes to isoproterenol and insulin

Insulin was added to the cells 24 hours prior to, and for 4 hours during, the lipogenesis assay. Different concentrations of isoproterenol were present throughout the 24 hour period to determine if it resulted in an increased response of the cells insulin-stimulation of lipogenesis. ANOVA showed a significant effect of isoproterenol ($P < 0.002$) and also of insulin ($P < 0.015$), as shown in Figure 6.3. However, the increase in lipogenesis in response to insulin was neither quantitatively (Figure 6.3a) nor proportionally (Figure 6.3b) different in cells exposed or not to isoproterenol.

6.3.2 Effects of various PDE inhibitors on lipogenesis in 3T3-F442A adipocytes

The 3T3-F442A adipocytes were incubated with various specific and non-specific PDE inhibitors. These were rolipram (Shakur *et al.*, 1995), a specific inhibitor of the PDE4 family; cilostamide (Tang *et al.*, 1994; Verghese *et al.*, 1995), a specific inhibitor of the PDE3 family; 8-methoxymethyl-IBMX (mm-IBMX) (Ahn *et al.*, 1989), a specific inhibitor of the Ca^{2+} / calmodulin-sensitive PDE1 family and also IBMX, which is a non-specific PDE inhibitor.

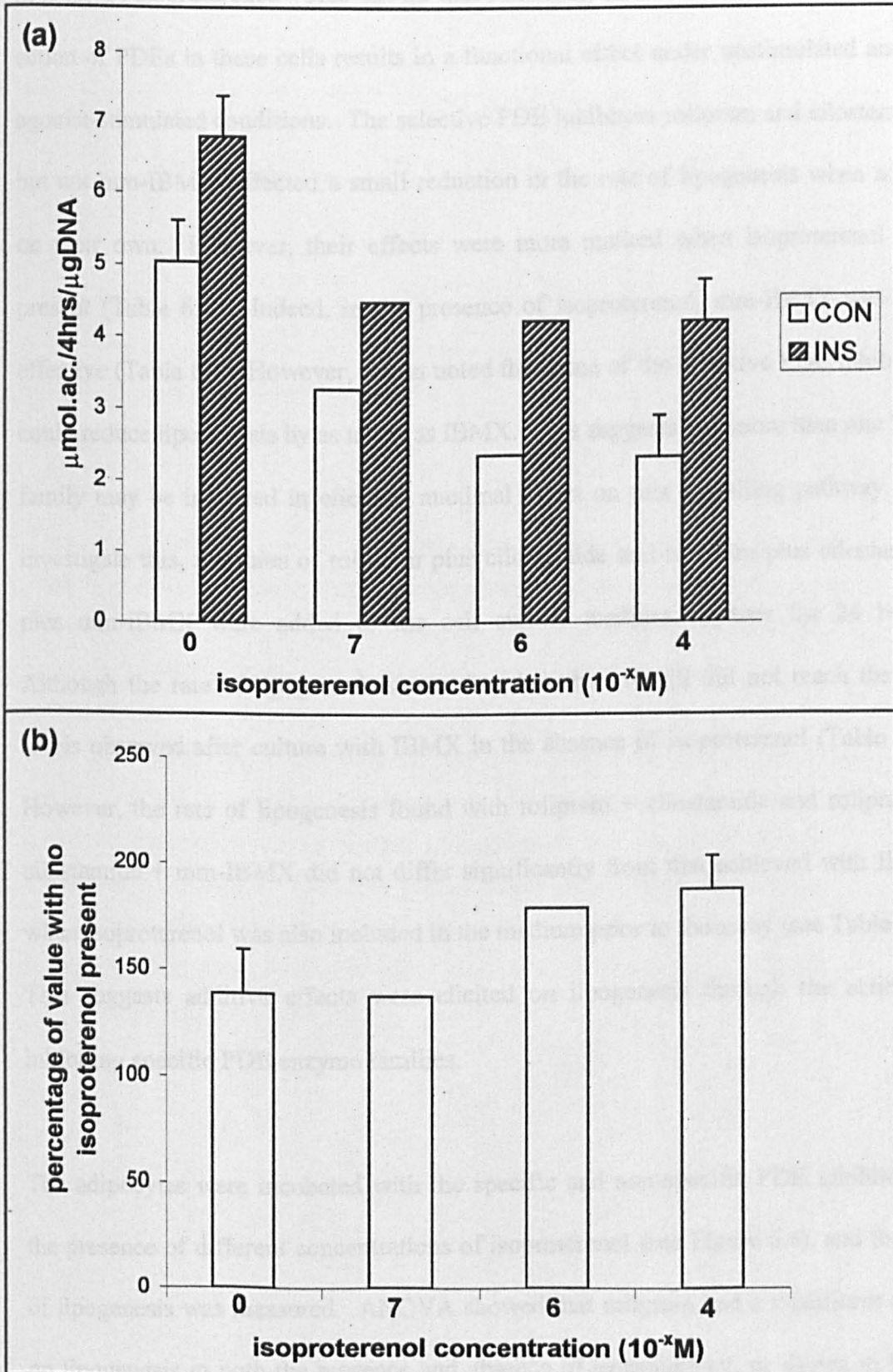
6.3.2.1 Different rates of lipogenesis in the presence or absence of isoproterenol

The differentiated 3T3-F442A adipocytes were incubated with the indicated PDE inhibitors in the presence or absence of isoproterenol for the 24 hour period prior to the lipogenesis assay. This was done in order to observe the effects of inhibiting

Figure 6.3. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5µg/ml), after which time the serum and insulin were removed from the medium. Different concentrations of isoproterenol were added to the medium 24 hours prior to, but not during, the lipogenesis assay. Insulin (INS ; 100ng/ml) was added 24 hours prior to, and during, the measurement of the rate of lipogenesis (CON = control), which was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period. (a) shows the absolute values for the rate of lipogenesis, and (b) shows the relative rate of insulin-stimulated lipogenesis. Results are for 4, 1, 2, 3 observations for isop concentrations 0, 10⁻⁷M, 10⁻⁸M, 10⁻⁴M, respectively, values for 0 and 10⁻⁴M isop are given + S.E.M.

Effect of isoproterenol on the stimulation of lipogenesis by

insulin in 3T3-F442A adipocytes



specific PDE family on lipogenesis. As shown in Table 6.1, the addition of IBMX resulted in the rate of lipogenesis being reduced by over 75% in either the presence or absence of isoproterenol. This shows that inhibiting cAMP breakdown through the action of PDEs in these cells results in a functional effect under unstimulated and β -agonist-stimulated conditions. The selective PDE inhibitors rolipram and cilostamide, but not mm-IBMX, effected a small reduction in the rate of lipogenesis when added on their own. However, their effects were more marked when isoproterenol was present (Table 6.1). Indeed, in the presence of isoproterenol, mm-IBMX was also effective (Table 6.1). However, it was noted that none of the selective PDE inhibitors could reduce lipogenesis by as much as IBMX. This suggests that more than one PDE family may be involved in eliciting maximal effect on this signalling pathway. To investigate this, mixtures of rolipram plus cilostamide and rolipram plus cilostamide plus mm-IBMX were added to the cell culture medium together for 24 hours. Although the rate of lipogenesis was reduced further, it still did not reach the low levels observed after culture with IBMX in the absence of isoproterenol (Table 6.1). However, the rate of lipogenesis found with rolipram + cilostamide and rolipram + cilostamide + mm-IBMX did not differ significantly from that achieved with IBMX when isoproterenol was also included in the medium prior to the assay (see Table 6.1). This suggests additive effects were elicited on lipogenesis through the action of inhibiting specific PDE enzyme families.

The adipocytes were incubated with the specific and non-specific PDE inhibitors in the presence of different concentrations of isoproterenol (see Figure 6.4), and the rate of lipogenesis was measured. ANOVA showed that rolipram had a significant effect on lipogenesis in both the presence and absence of isoproterenol, as shown in figure

The effect of different PDE inhibitors on the rate of lipogenesis
when isoproterenol was absent or present

	PDE inhibitor						mm+rol+ cil
	control	ibmx	rol	cil	mm	rol+cil	
no isop	3.68 ^a	0.81 ^b	2.78 ^c	2.9 ^c	3.24 ^{ac}	2.03 ^d	1.68 ^d
isop10 ⁻⁴ M	1.82 ^a	0.4 ^b	0.88 ^{cd}	1.19 ^c	1.16 ^c	0.72 ^{bc}	0.59 ^{bd}

rate of lipogenesis ($\mu\text{mol.ac/4hrs/mgDNA}$)

Table 6.1. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5 $\mu\text{g/ml}$), after which time the serum and insulin were removed from the medium. Different selective and non-selective PDE inhibitors (control = no PDE inhibitor, rol = rolipram (10 μM) (Shakur *et al.*, 1995), cil = cilostamide (10 μM) (Verghese *et al.*, 1995; Tang *et al.*, 1994), mm = methoxymethyl-IBMX (10 μM) (Ahn *et al.*, 1989) and IBMX (1mM)) were added to the medium, with or without isoproterenol (isop ; 10⁻⁴M), for 24 hours prior to, but not during, the measurement of the rate of lipogenesis. This was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period. Results are the means of 6 observations, S.E.M.= 0.2604 for no isop' and S.E.M. = 0.1566 for '+ isop'. Values in a row without the same suffix, a,b,c,d, differ significantly, P<0.05.

The effect of different selective PDE inhibitors on the rate of lipogenesis when various concentrations of isoproterenol are present

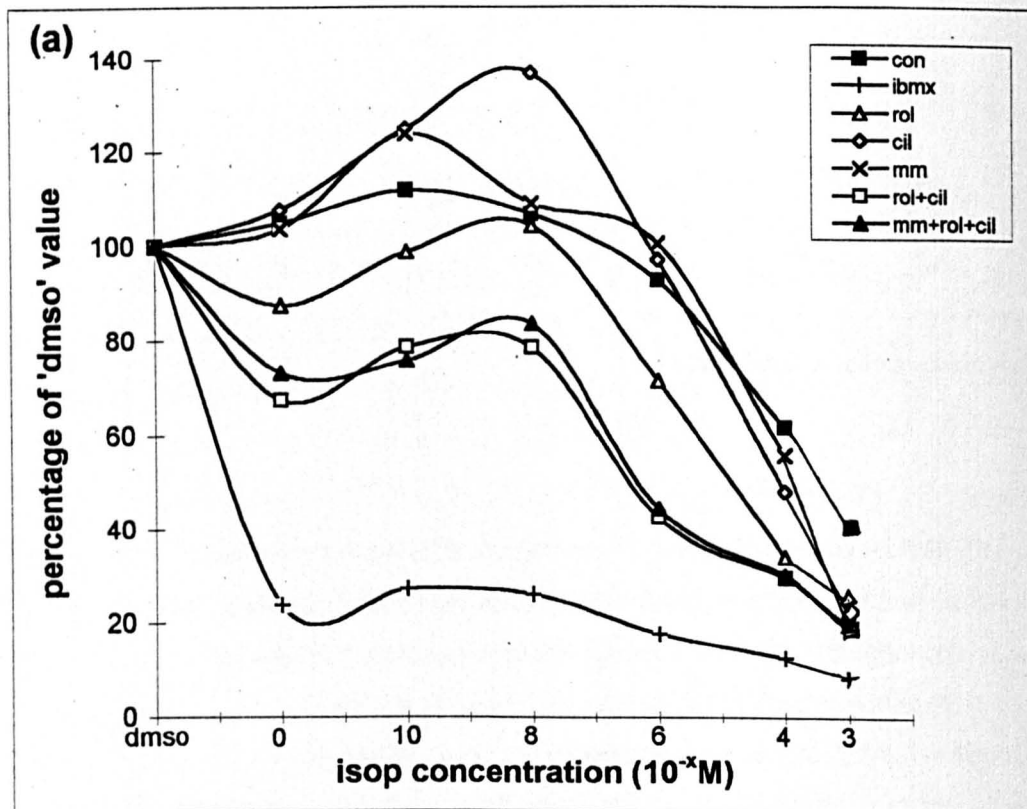
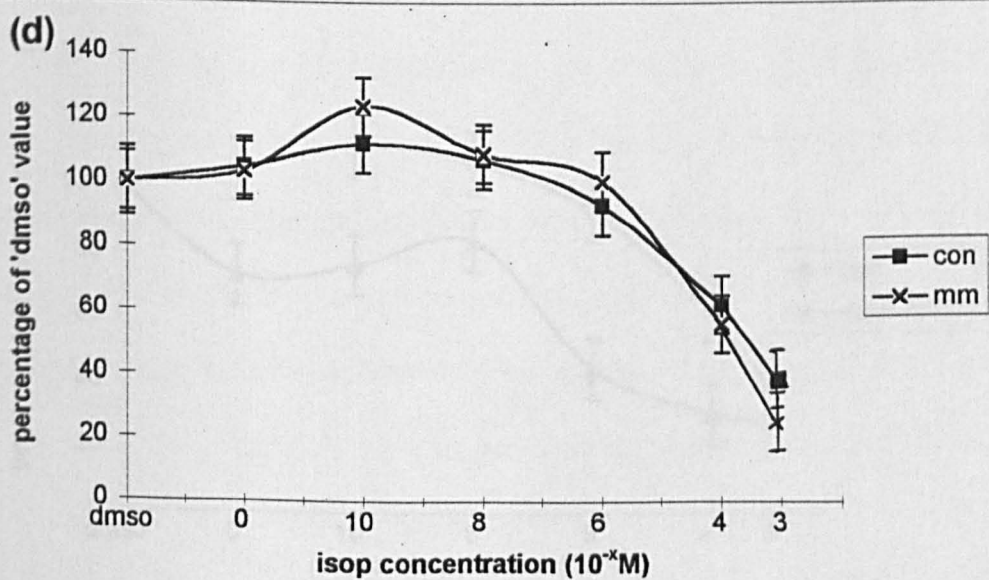
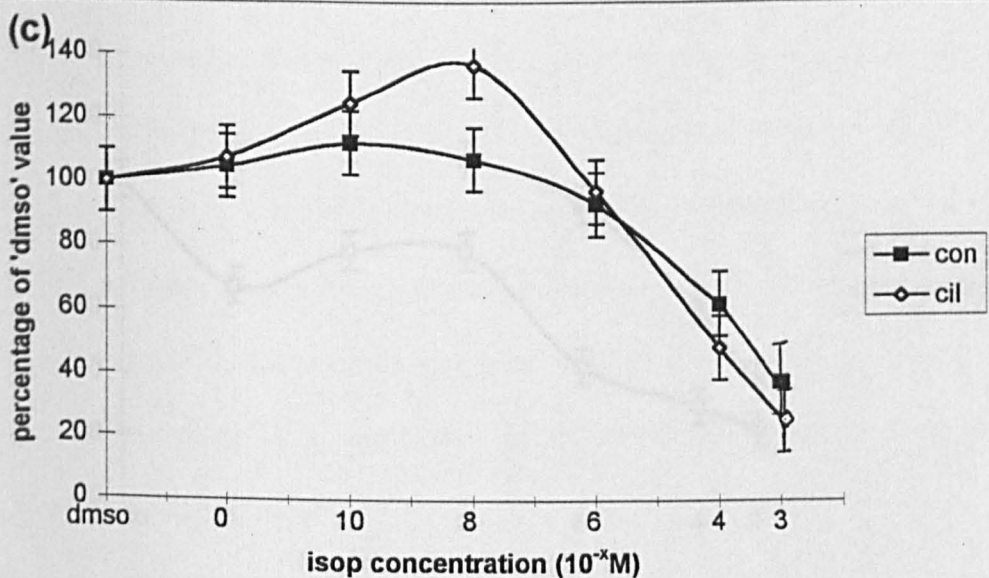
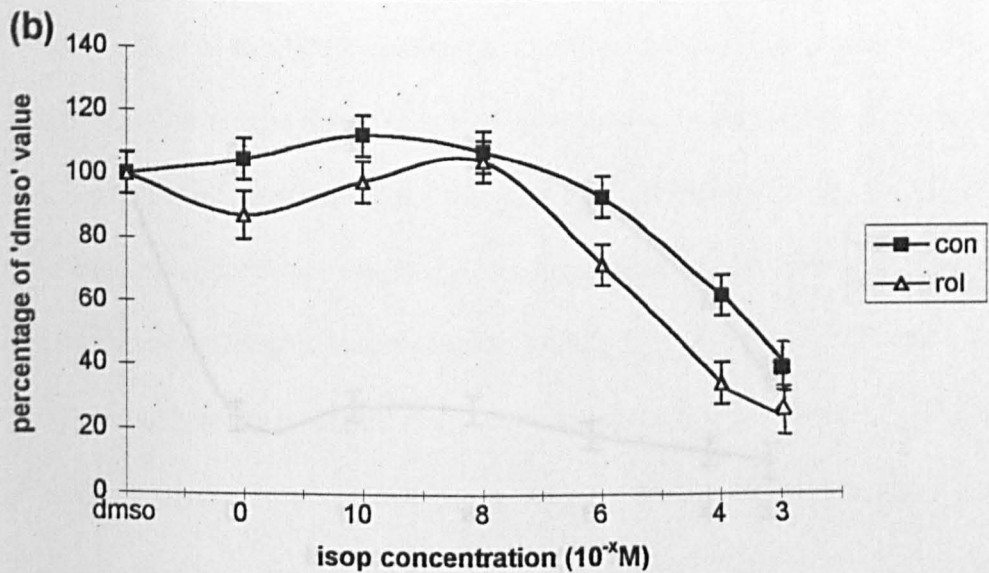
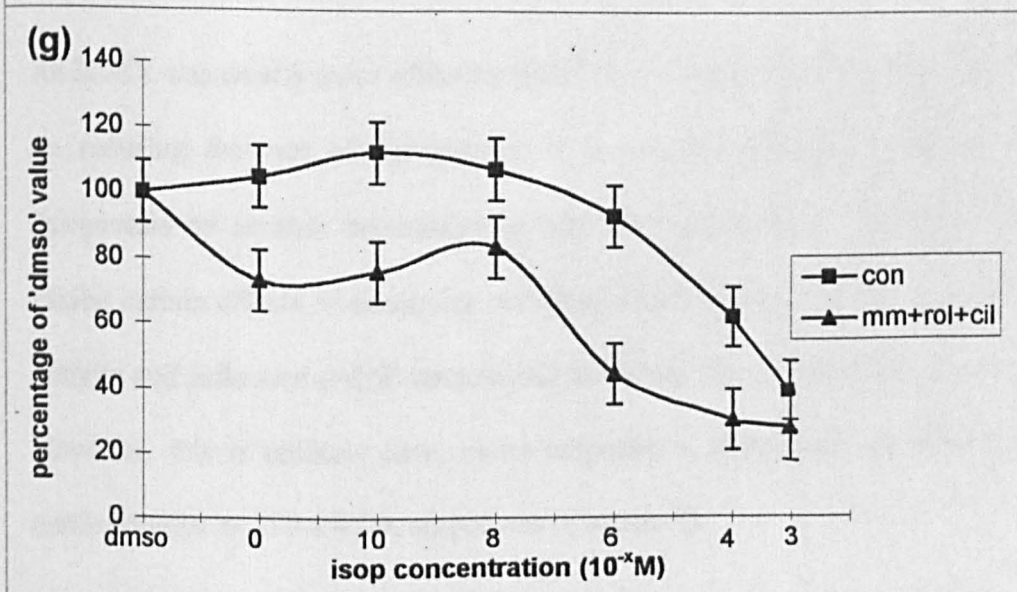
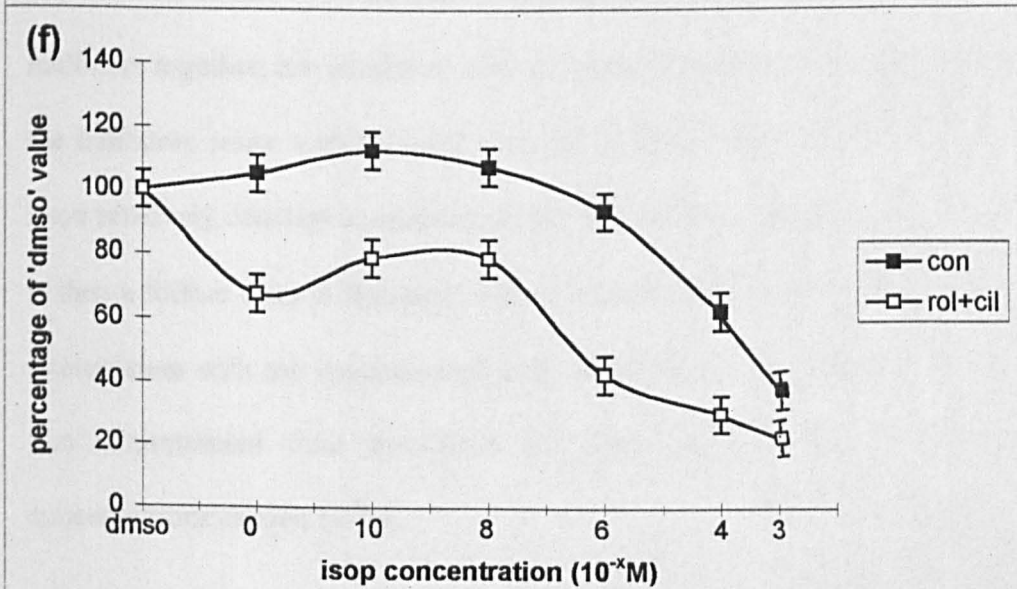
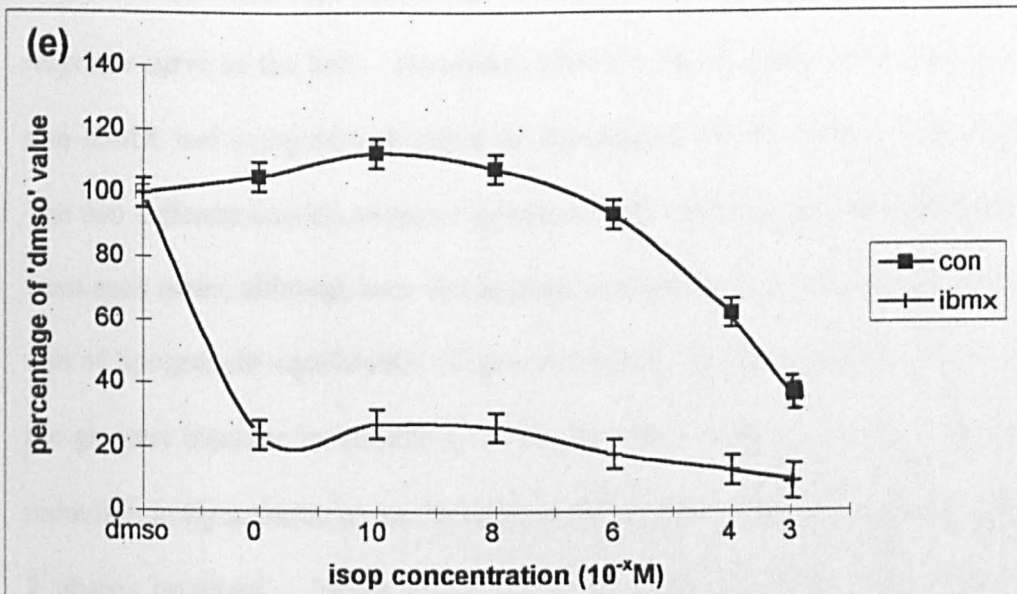


Figure 6.4. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5µg/ml), after which time the serum and insulin were removed from the medium. Various concentrations of isoproterenol were added to the medium for 24 hours prior to, but not during, the measurement of the rate of lipogenesis, together with different selective PDE inhibitors (con = no PDE inhibitor, rol = rolipram (10µM), cil = cilostamide (10µM), mm = 8-methoxy-IBMX (10µM)) and the non-selective PDE inhibitor IBMX (1mM). The rate of lipogenesis was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period. Results are the means of 4 observations, and are standardised as a percentage of the dms0 control value. (b)-(g) are +/- S.E.M. derived from ANOVA.





6.4(b) and increased the sensitivity to isoproterenol moving the isoproterenol dose response curve to the left. However, ANOVA showed that neither cilostamide nor mm-IBMX had a significant effect on lipogenesis in this study (Figures 6.4(c)(d)). The two different combinations of selective PDE inhibitors did not differ significantly from each other, although they did increase sensitivity to isoproterenol and reduce the rate of lipogenesis significantly (Figure 6.4(f)(g)). However, IBMX alone resulted in the greatest increase in sensitivity to isoproterenol (Figure 6.4(e)). The pattern of reduction in lipogenesis in the presence of isoproterenol shows that there appear to be 2 phases involved. When either rolipram or IBMX alone, or the selective PDE inhibitors together, are incubated with the adipocytes in the absence of isoproterenol, the inhibitors cause a reduction in the rate of lipogenesis. This level of lipogenesis stays relatively constant as isoproterenol concentrations increase, until 10^{-8} M. There is then a further drop in lipogenic rate as isoproterenol concentration increases. This is consistent with the isoproterenol dose response curve in Figure 6.1, which shows that isoproterenol dose not have an effect on the rate of lipogenesis until concentrations exceed 10^{-8} M.

As IBMX was clearly more effective than the combination of all three PDE inhibitors on reducing the rate of lipogenesis, it is possible it may be having effects on lipogenesis by another mechanism in addition to inhibition of PDEs. IBMX can inhibit certain effects of adenosine and thus, under certain conditions, could alter AC activity and influence cAMP content and lipolysis / lipogenesis (Londos *et al.*, 1978). However, this is unlikely here, as no response to adenosine has been observed in earlier studies in 3T3-F442A adipocytes (Chapter 5).

6.3.3 Different concentrations of selective and non-selective PDE inhibitors

6.3.3.1 IBMX dose response data

The effect of different concentrations of IBMX on lipogenesis was observed in the absence of isoproterenol. Figure 6.5 shows that the concentration routinely used in other experiments (1mM) resulted in the greatest reduction in lipogenesis, with the rate decreasing as IBMX concentration increased.

6.3.3.2 Selective PDE inhibitor dose response data

The 3T3-F442A adipocytes were chronically incubated with a range of concentrations of selective PDE inhibitors, as shown in Figure 6.6, to investigate the effect on lipogenesis. Earlier literature had suggested that 10 μ M was the optimum concentration of each of the PDE inhibitors. Higher concentrations of both rolipram and mm-IBMX had no further effect on the rate of lipogenesis. However, it appeared that higher concentrations than those routinely used of cilostamide reduced the rate of lipogenesis further in the presence of isoproterenol. This suggests that higher concentrations of cilostamide were required to be present to fully inhibit the PDE3 enzyme. However, higher concentrations of cilostamide may be toxic to the cell or affect other components within the cells.

6.3.4 Chronic and acute incubation of PDE inhibitors with 3T3-F442A adipocytes

IBMX, rolipram, cilostamide and mm-IBMX were added to the medium, either in a mixture or separately, for either 24 hours or 2 hours prior to the measurement of the rate of lipogenesis. Isoproterenol (10⁻⁴M) was present for 24 hours prior to the assay. The PDE inhibitors were also either present or absent during the assay, as shown in

Figure 6.7. ANOVA shows a significant effect of time ($P < 0.023$) and also of PDE inhibitor ($P < 0.0001$) on the rate of lipogenesis. For the untransformed data shown in Figure 6.7a, the lowest overall rate of lipogenesis was found after exposure to the inhibitors for 24+4 hours, and the greatest rate of lipogenesis was found after 2 hours or 2+4 hours, with 24 hours culture being intermediate. IBMX had the most effect on the rate of lipogenesis, with the selective PDE inhibitors alone having a small effect and the combinations being intermediate. Significant effects of time ($P < 0.0001$) and PDE inhibitor ($P < 0.0001$) were also found when the relative rates of lipogenesis were compared, as shown in Figure 6.7b. The PDE inhibitors were required to be present for over 2 hours, and the lowest rate of lipogenesis was observed after either 24+4 hours or 2+4 hours.

The effect of different concentrations of IBMX on the basal rate of lipogenesis

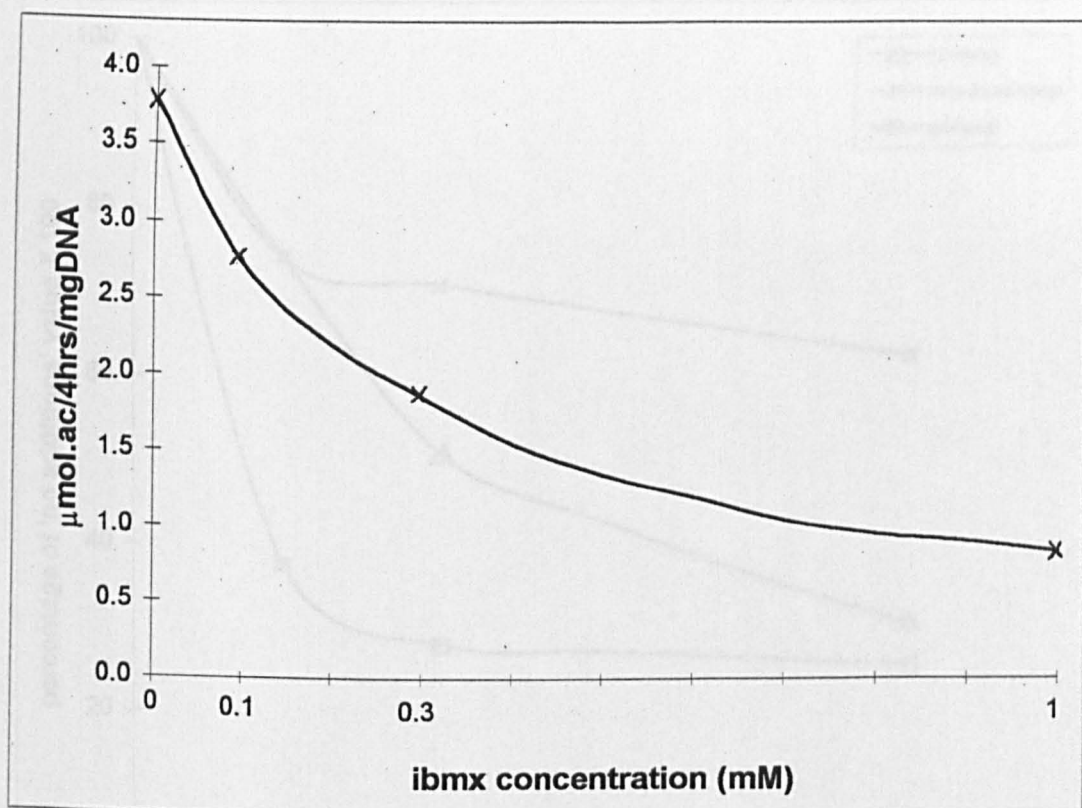


Figure 6.5. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5μg/ml), after which time the serum and insulin were removed from the medium. Various concentrations of the non-selective PDE inhibitor IBMX were added to the medium for 24 hours prior to, but not during, the measurement of the rate of lipogenesis. This was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period. Results are the means of 2 observations.

The effect of different PDE inhibitor concentrations on the rate of lipogenesis

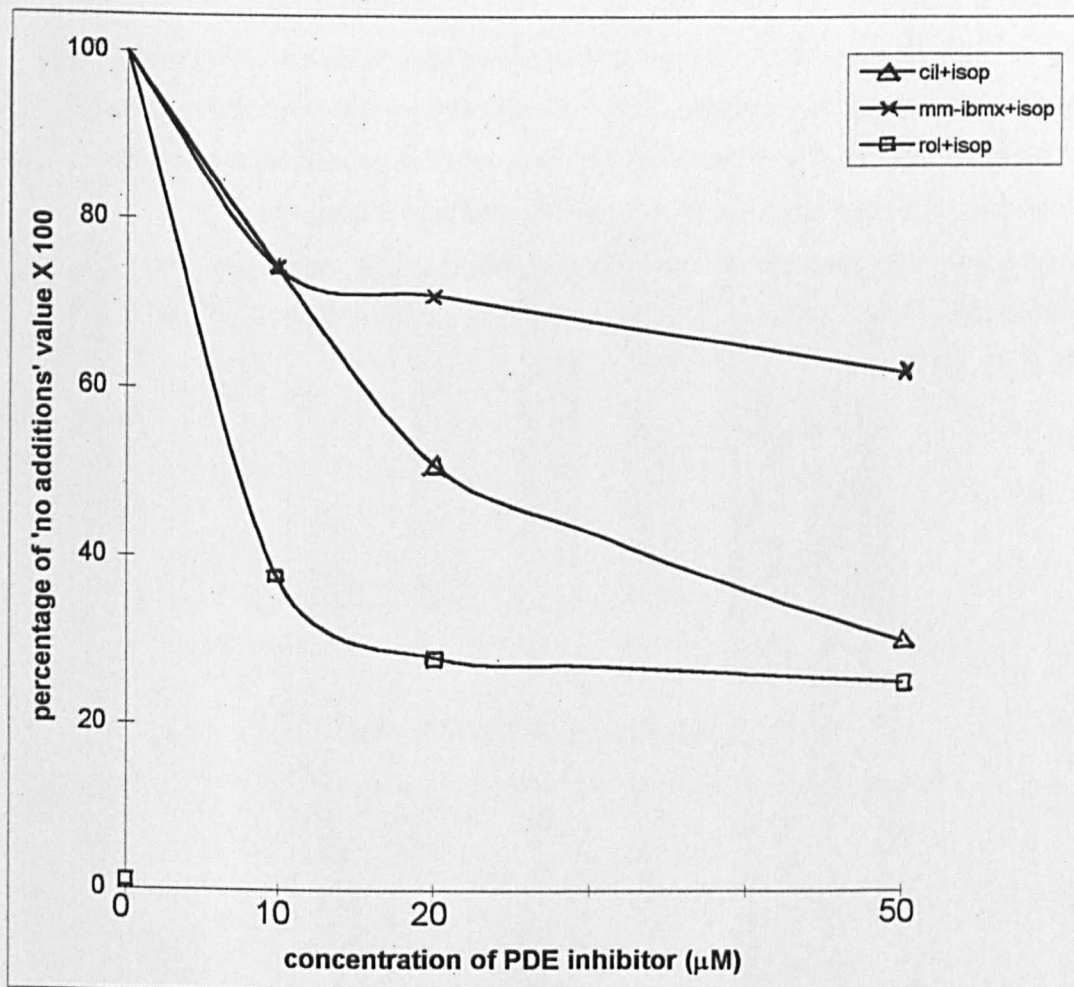
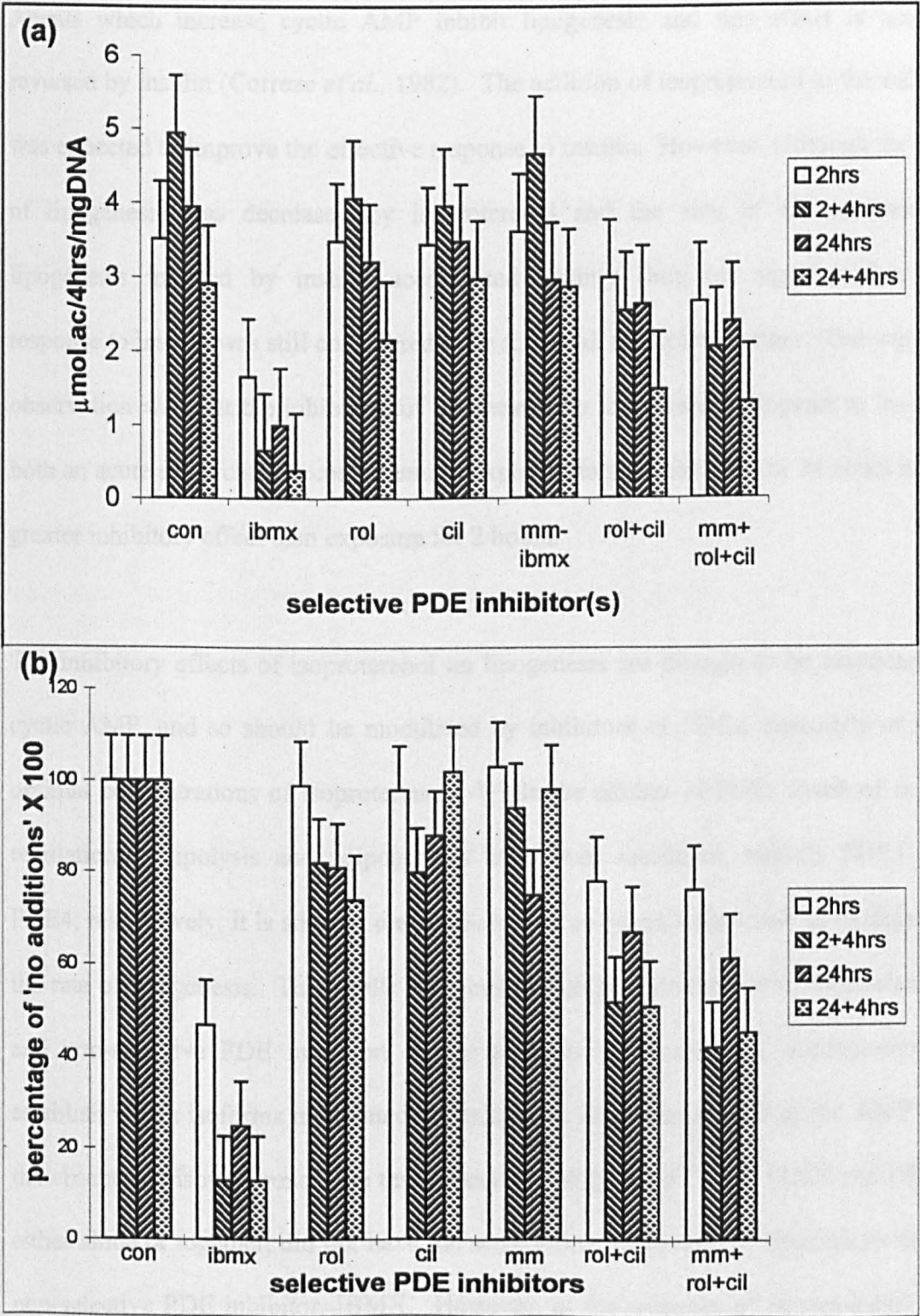


Figure 6.6. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin ($5\mu\text{g/ml}$), after which time the serum and insulin were removed from the medium. Various concentrations of selective PDE inhibitor (with isoproterenol at a concentration of 10^{-4}M) were added to the medium 24 hours prior to, but not during, the measurement of the rate of lipogenesis (isop = isoproterenol, rol = rolipram, cil = cilostamide, mm = methoxymethyl-IBMX). This was determined by measuring the amount of ^{14}C -labelled sodium acetate incorporated into cell lipid over a 4 hour period. Results are the means of 2 observations.

Figure 6.7. 3T3-F442A pre-adipocytes were differentiated for 8 days in medium containing serum and insulin (5µg/ml), after which time the serum and insulin were removed from the medium. Selective PDE inhibitors (con = control, rol = rolipram (10µM), cil = cilostamide (10µM), mm = methoxymethyl-IBMX (10µM)) and the non-selective PDE inhibitor IBMX (1mM) were added to the medium for either 24 hours or 2 hours prior to the measurement of the rate of lipogenesis (isoproterenol (10⁻⁴M) was present for 24 hours prior to the assay). The PDE inhibitors were also either present or absent during lipogenesis, the rate of which was determined by measuring the amount of ¹⁴C-labelled sodium acetate incorporated into cell lipid over a 4 hour period. (a) shows the absolute values for the rate of lipogenesis, + S.E.M., while (b) shows the relative rate of lipogenesis, + S.E.M. Results are the means of 3 observations.

The effect of different PDE inhibitors when they are present for various periods prior to, or during, the measurement of the rate of lipogenesis



6.4 Discussion

Insulin-stimulation of lipogenesis in 3T3-F442A adipocytes observed previously (Chapter 5) was not as large as expected, so it was decided to subject the adipocytes to exposure to isoproterenol prior to measuring the insulin stimulation of lipogenesis. Agents which increase cyclic AMP inhibit lipogenesis and this effect is usually reversed by insulin (Correze *et al.*, 1982). The addition of isoproterenol to the culture was expected to improve the effective response to insulin. However, although the rate of lipogenesis was decreased by isoproterenol and the size of the increase in lipogenesis induced by insulin accentuated slightly (but not significantly), the response to insulin was still considered to be too small to explore further. One curious observation was that the inhibition of lipogenesis by isoproterenol appears to involve both an acute and a chronic component as exposure to isoproterenol for 24 hours had a greater inhibitory effect than exposure for 2 hours.

The inhibitory effects of isoproterenol on lipogenesis are thought to be mediated by cyclic AMP, and so should be modulated by inhibitors of PDEs, especially at sub-optimal concentrations of isoproterenol. While the classes of PDEs involved in the regulation of lipolysis and adipogenesis have been identified, namely PDE3 and PDE4, respectively, it is still not clear which PDE enzymes have a role in modulating the rate of lipogenesis. Thus, cells were cultured in the presence of various selective and non-selective PDE inhibitors in the presence or absence of isoproterenol to establish which isoforms modulated the inhibition of lipogenesis by cyclic AMP. In the absence of isoproterenol, the three specific inhibitors of PDE1, PDE3 and PDE4, either alone or together, did not have the same effect on the rate of lipogenesis as the non-selective PDE inhibitor, IBMX. However, in the presence of isoproterenol, the

three specific PDE inhibitors all had some effect on inhibition of lipogenesis, and the combination of either PDE3 and PDE 4 enzymes inhibitors, or PDE1, 3 and 4 enzyme inhibitors, reduced the rate of lipogenesis to levels which did not differ significantly from that achieved with IBMX, as shown in Table 6.1 and Figure 6.4.

The pattern of reduction in lipogenesis in the presence of isoproterenol shows that there appear to be 2 phases involved. The incubation of the PDE inhibitors with adipocytes in the presence of different concentrations of isoproterenol appeared to involve a biphasic reduction in lipogenesis. When either rolipram or IBMX alone, or the selective PDE inhibitors together, are incubated with the adipocytes in the absence of isoproterenol, the inhibitors cause a reduction in the rate of lipogenesis. This level of lipogenesis stays relatively constant as isoproterenol concentrations increase, until 10^{-8} M. There is then a further drop in lipogenic rate as isoproterenol concentration increases. This is consistent with the isoproterenol dose response curve in Figure 6.1, which shows that isoproterenol dose not have an effect on the rate of lipogenesis until concentrations exceed 10^{-8} M.

Both PDE3B and PDE4 enzymes are found in adipocytes, and have been implicated in signalling pathways in adipocytes, with PDE4 being considered important in adipocyte differentiation, and PDE3 being an important factor for lipolysis (Elks & Manganiello, 1985; MacKenzie *et al.*, 1998). It has been suggested that there is compartmentalisation of cAMP signalling between a pool controlled by PDE3 activity and another pool controlled by PDE4 activity, as it has been observed that there were differences in response to selective PDE inhibitors for these isoforms in adipocytes (Elks & Manganiello, 1985; MacKenzie *et al.*, 1998) and other cell types (Faux &

Scott, 1996; Erdogan & Houslay, 1997; Houslay & Milligan, 1997). However, while PDE3 appears to be the most important isoform of PDE involved in the regulation of lipolysis, it does not appear to be the only isoform involved. Arner and colleagues (1993) showed that inhibition of PDE3 stimulated lipolysis, but not to the same extent as a non-selective PDE inhibitor, suggesting that other PDE isoforms were involved in lipolysis regulation. In contrast to lipolysis, inhibition of PDE1, PDE3 and PDE4 isoforms appears to be equally effective in accentuating the inhibition of lipogenesis by isoproterenol, and the combination of all three inhibitors achieved a similar effect on lipogenesis as the non-specific PDE inhibitor, IBMX, in the presence of isoproterenol. In the absence of isoproterenol, IBMX was clearly much more effective than the combination of all three inhibitors, so may be having effects on lipogenesis by another mechanism in addition to inhibition of PDEs. IBMX can inhibit certain effects of adenosine and thus, under certain conditions, could alter AC activity and influence cAMP content and lipolysis / lipogenesis (Londos *et al.*, 1978). However, this is unlikely here, as no response to adenosine has been observed in earlier studies in 3T3-F442A adipocytes (Chapter 5).

Thus the pool of cyclic AMP involved in the inhibition of lipogenesis may not be the same as that involved in the stimulation of lipolysis by catecholamine. Lipolysis is under acute control by catecholamines, whereas the effects on lipogenesis appear to involve both acute and chronic components, which is consistent with the two metabolic processes being modulated by different pools of cyclic AMP. The basis of the apparent chronic effect of isoproterenol on lipogenesis in the 3T3-F442A adipocytes was not determined, but could be due to a change in the amount of one or more lipogenesis enzymes. Agents which increase cyclic AMP levels decreased the

expression of lipogenic enzyme genes in rat adipose tissue *in vitro* (Foufelle *et al.*, 1994) and also in differentiating 3T3-F442A (Spiegelman & Green, 1981) and 3T3-L1 adipocytes (Weiss *et al.*, 1980 ; Paulauskis & Sul, 1988 ; Moustaid & Sul, 1991). The studies by Spiegelman and Green (1981) also showed that cyclic AMP could inhibit lipogenic enzyme gene expression by a mechanism which did not involve lipolysis. This observation is consistent with different pools of cyclic AMP modulating lipolysis and lipogenesis.

The identity of the PDE isoforms involved in mediating aspects of insulin stimulation of lipogenesis in the 3T3-F442A adipocytes remains to be investigated. It has already been shown that PDE3B activation / phosphorylation is inhibited by the PI-3K inhibitor wortmannin, thus blocking the antilipolytic action of insulin (Okada *et al.*, 1994). Wijkander and colleagues (1998) have shown that PKB is responsible for the phosphorylation of PDE3B *in vitro*, but insulin-induced activation of p70 S6 kinase and MAP kinases are not involved in its activation (Wijkander *et al.*, 1998). Further investigation is required into the involvement of specific PDE isoforms in insulin signalling to help elucidate this complex pathway in adipocytes.

CHAPTER SEVEN

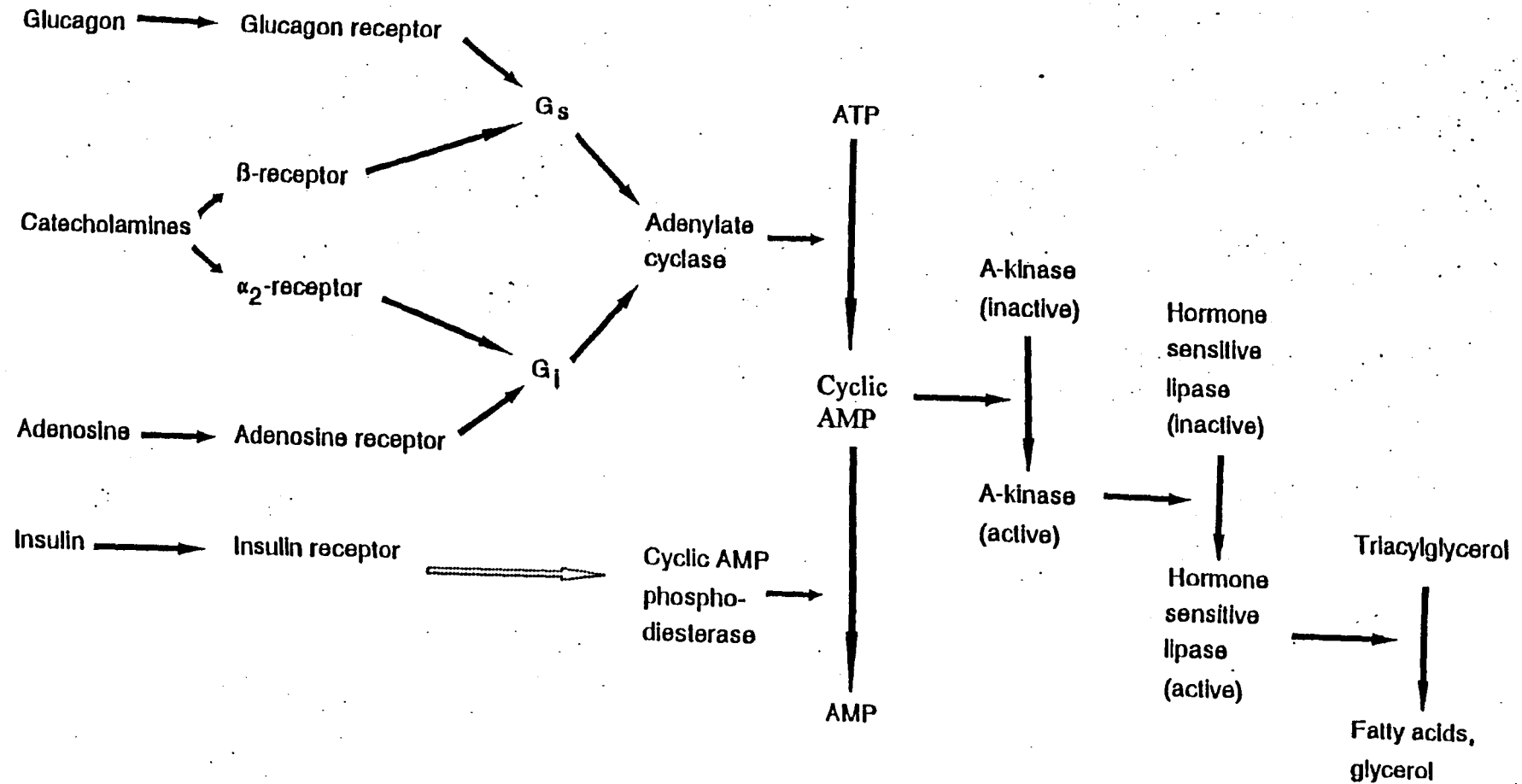
GENERAL DISCUSSION

General Discussion

It has become increasingly clear that technological developments in a variety of scientific and engineering fields will be required to support the ever increasing world population, which is estimated to double over the next four decades (Bauman, 1992). The need for technological innovations in agriculture production systems is clear. Because the GH-mediated changes in adipose tissue metabolism play a pivotal role in accounting for the effects of GH on animal growth and carcass composition, considerable effort has been made to increase our understanding of how GH alters nutrient utilisation in adipose tissue. The expansion of our knowledge of GH has enabled scientists to conceptualise and develop strategies to modify carcass composition, improve feed efficiency, enhance growth rate and milk yield, while concurrently decreasing adipose tissue accretion (for review, see Etherton & Bauman, 1998).

Food energy in animals is stored primarily as triacylglycerols in lipid storage droplets of adipocytes. The important features of adipocyte metabolism are the synthesis, storage and subsequent hydrolysis of these triacylglycerols, and the relative rates of their synthesis and breakdown determine the amount of lipid within the adipocyte. The release of fatty acids (lipolysis) to fulfil metabolic needs during periods of negative energy balance is under complex acute and chronic endocrine control (Vernon, 1992). The crucial step in triacylglyceride breakdown is activation of the HSL, which is mediated by the cyclic AMP and the AC system, and the subsequent cascade of protein phosphorylation (Figure 7.1). Lipolysis is stimulated acutely by catecholamines acting *via* β -adrenergic receptors and glucagon acting *via* the

Figure 7.1 Endocrine control of lipolysis



glucagon receptor. It is inhibited by adenosine acting *via* the adenosine A₁ receptor and also catecholamines acting *via* the α 2-adrenergic receptor.

Acute, insulin-like effects of GH have been observed in some species (not sheep), but these effects have been controversial and their physiological significance uncertain. Chronic treatment with GH seems to increase the rate of lipolysis by affecting both the inhibitory and stimulatory systems. Initial studies showed that chronic treatment with GH in sheep adipose tissue explants *in vitro* increased the response to catecholamine, thereby increasing the rate of isoproterenol-stimulated lipolysis, which is consistent with the findings of Watt *et al.* (1991). A decrease in response to PIA was also observed in sheep adipose tissue explants after chronic culture with GH, which had previously been found (Vernon *et al.*, 1991b; Doris *et al.*, 1996, 1998).

GH also chronically decreases adiposity in animals by having insulin-antagonistic effects resulting in a decrease in lipogenesis, both *in vivo* and *in vitro* (Nyberg & Smith, 1977; Vernon, 1982; Walton & Etherton, 1986; Etherton *et al.*, 1987) (Chapter 1, section 1.3.3). However, the signalling system involved in the chronic, insulin-antagonistic effect of GH on lipogenesis is not known.

Inhibitors of different proteins involved in various signalling pathways have been used to try and determine the mode of action of the chronic effects of GH (Table 7.1). Studies with sheep adipocytes using inhibitors such as rapamycin and PD 98059 suggest that neither p70^{S6K} nor MAP kinase are involved with chronic related effects of GH (see section 1.3.3). However, anti-lipogenic, insulin-antagonistic effects of GH appear to involve both protein serine kinases and phosphatases (Vernon, 1996) by use

**Effects of some inhibitors on attenuation of lipogenesis and the antilipolytic effects of PIA by growth hormone
in sheep adipose tissue**

Inhibitor	Target	GH inhibition of lipogenesis	GH inhibition of antilipolytic effect of PIA
Actinomycin D	gene transcription	prevented	prevented
H7	serine kinases	prevented	prevented
Okadaic acid	serine phosphatases	prevented	mimicked
Wortmannin	PI 3-kinase	mimicked	mimicked
PD 98059	MAP kinase	no effect	no effect
PMA (chronic)	PKC isoforms	partially prevented	mimicked

Table 7.1. Explants of adipose tissue from 6 month old, castrated, male sheep were maintained in culture for 22 hours in the absence of exogenous hormones and inhibitors, and then for a further 24 hours in the presence and absence of 4.5nM growth hormone plus or minus an inhibitor. Subsequently, the rate of lipogenesis or response to PIA were assessed (Chapter 2, section 2.2.3).

From Vernon (1996), Doris *et al.* (1998) and unpublished observations.

of the inhibitors H7, staurosporine and okadaic acid. It has been shown that the loss of the acute, insulin-like effects of GH and the development of enhanced lipolysis in rat adipocytes are both prevented by the addition of the transcription inhibitor, Actinomycin D (Goodman *et al.*, 1987; Goodman, 1993). Borland *et al.* (1994) found that the inhibition of lipogenesis in sheep adipose tissue by GH was prevented by Actinomycin D and required polyamines, effects of which include a role in gene transcription (Scalabrino *et al.*, 1991; Sjöholm, 1993), activation of a number of protein kinases and phosphatases (Morgan, 1990) and enhancing hormone binding to plasma membrane receptors (Pedersen *et al.*, 1989). At least one isoform of PKC has also been shown to be involved in GH inhibition of lipogenesis as pretreatment with phorbol ester (PMA), which down-regulates PKC, partially prevented the GH effect. PKC has also been suggested as being involved in GH inhibition of the anti-lipolytic effect of adenosine (Doris *et al.*, 1998).

The studies by Borland *et al.* (1994) which found that the inhibition of lipogenesis in sheep adipose tissue by GH required polyamines, and that the product(s) of gene transcription must have a very short half-life (less than 3 hours) prompted the investigation into whether the cytokine TNF α could be this putative protein. TNF α had been shown to chronically increase the rate of basal lipolysis in rat epididymal adipocytes (Green *et al.*, 1994; Gasic *et al.*, 1999), an effect similar to that of GH, and therefore was a possible candidate to mediate the action of GH. TNF α also has insulin antagonistic effects. The mitogenic effects of GH are mediated by IGF-1, and there was the possibility that chronic metabolic effects of GH could be by an analogous mechanism. However, although TNF α did result in a small increase in basal lipolysis in sheep and rat adipose tissue explants, it had no effect on the rate of isoproterenol-

stimulated lipolysis, unlike GH. TNF α did show a tendency to decrease the response to adenosine in sheep and rat explants (consistent with Gasic *et al.*, 1999), but the effect was very small.

Increasing the proximity to the lymph nodes embedded in guinea pig popliteal adipose tissue had previously been shown to result in an increase in lipolysis when cultured with lymph node lymphoid cells (Pond & Mattacks, 1995; Mattacks & Pond, 1997), so it was decided to investigate if the effects of TNF α on lipolytic rate in sheep popliteal tissue could be enhanced by using tissue from near to the lymph node. However, although adipose tissue from near to the lymph node showed a smaller increase in response to isoproterenol and diminished response to adenosine, there were no differences in the ability of TNF α to stimulate basal or catecholamine-stimulated lipolysis.

However, TNF α did cause a reduction in insulin stimulation of lipogenesis in ovine subcutaneous tissue explants, although it did not have any effects by itself. This is consistent with previous findings which suggest that the cytokine has a role in the induction of a generalised state of insulin resistance. In 3T3-F442A adipocytes, TNF α inhibits enzymes involved in fat synthesis (Pekala *et al.*, 1983; Torti *et al.*, 1985; Semb *et al.*, 1987; Pape & Kim, 1988), and results in peripheral insulin resistance in rats (Lang *et al.*, 1992) and humans (Van der Poll *et al.*, 1991). However, the role of TNF α in regulating adipocyte lipolysis and promoting insulin resistance *in vivo* is still controversial, and some of the findings published have been contradictory (refer to Chapter 3). Although I found that the effects of TNF α on basal

lipolysis in rats and sheep were similar to those found by Green *et al.* (1994), and TNF α had similar effects to GH on inhibition of insulin-stimulated lipogenesis, TNF α did not mimic all the effects of GH on adipose tissue metabolism, and its effects were smaller than those of GH. I therefore concluded that TNF α was not the putative secreted protein involved in GH regulation of lipolysis and lipogenesis in adipose tissue.

It has been found that chronic exposure to phorbol ester (PMA) partly attenuated the antilipogenic effect of GH in sheep adipocytes, suggesting the involvement of one or more isoforms of PKC (Vernon, 1996). PKC is a family of phospholipid-dependent kinases involved in basic cellular functions, including regulation of growth, differentiation and gene expression (Pears *et al.*, 1992; Nishizuka, 1995). The role of individual PKC's in these processes is not yet known, although it seems likely that individual PKC's have unique rather than overlapping functions as most cells express more than one type of PKC.

All of the PKC's require phosphatidylserine for maximal activity. However, PKC's can be grouped according to differences in their dependence on other activators. In addition to phosphatidylserine, conventional PKC's require calcium and diacylglycerol (DAG) and consist of PKC α , β I, β II and γ (Coussens *et al.*, 1986; Knopf *et al.*, 1986; Parker *et al.*, 1986), novel PKC's require only DAG and are insensitive to calcium (PKC δ , ϵ , η , θ) (Ono & Nishizuka, 1987; Ohno *et al.*, 1988; Bacher *et al.*, 1991; Osada *et al.*, 1992), and atypical PKC's (ζ , ι/λ) are thought to require nothing more but phospholipid (Ono *et al.*, 1989; Selbie *et al.*, 1993; Akimoto

et al., 1994). There also exists PKC μ (Johannes *et al.*, 1994) and PKD (Valverde *et al.*, 1994), which are thought to be human/murine homologues, and are structurally quite different from the other isoforms; therefore, they may represent a new PKC sub-family. PKC isoforms display distinct regulatory properties and tissue distributions (Borner *et al.*, 1992; Hata *et al.*, 1993; Hug & Sarre, 1993; Selbie *et al.*, 1993; Stabel & Parker, 1993; Akimoto *et al.*, 1994; Hirai *et al.*, 1994; Dieterich *et al.*, 1996), which is consistent with individual isoforms probably serving diverse functional roles.

PKC has been implicated in having a role in some of the signalling events mediated by GH (see Figure 7.2). GH increases cellular levels of DAG in various cells (Doglio *et al.*, 1989; Catalioto *et al.*, 1992). Inhibition/down-regulation of PKC by prolonged exposure of cells to PMA resulted in attenuation of the activation of MAP kinases and two S6 kinases by GH in 3T3-F442A pre-adipocytes (Anderson, 1992, 1993). GH requires specific PKC isoforms to activate MAP kinases in 3T3-F442A pre-adipocytes (MacKenzie *et al.*, 1997) and evidence suggests that PKC lies downstream of PI-3kinase and upstream of MAP kinases (Kilgour *et al.*, 1996; MacKenzie *et al.*, 1997) and p70^{S6} kinase (Anderson *et al.*, 1992, 1993) in the signalling pathways utilised by GH in 3T3-F442A pre-adipocytes. PKC isoforms have also been implicated in being involved in the cell differentiating effects of GH (Gurland *et al.*, 1990; Campbell *et al.*, 1992; Ailhaud *et al.*, 1992; Catalioto *et al.*, 1992). PKC also mediates the GH signal transduction pathways resulting in inhibition and stimulation of lipogenesis and lipolysis in adipocyte metabolism (Smal & De Meyers, 1987; Gorin *et al.*, 1990; Vernon, 1996), and appears to be involved in mediating GH reduction in sensitivity to adenosine of sheep adipose tissue (Vernon, 1996; Doris *et al.*, 1998). However, the

effects are different. In lipogenesis, PMA partly prevents the effects of GH, whereas PMA mimics the effect of GH on the antilipolytic effect of PIA.

While inhibitor studies implicate isoforms of PKC, they cannot distinguish which ones are involved. Other studies in the laboratory were using an antisense approach to eliminate specific isoforms of PKC to determine their role in the differentiation of 3T3-F442A pre-adipocytes (Fleming *et al.*, 1998). This approach was subsequently used to show that PKC γ and PKC μ were involved in the chronic effect of GH on lipogenesis in differentiated 3T3-F442A adipocytes (Millar, 1998). Hence, I decided to try an use that approach to determine the isoforms involved in the GH effects on the antilipolytic effect of PIA. To investigate the involvement of specific PKC isoforms in the reduction of sensitivity of sheep adipocytes to adenosine in isoproterenol-stimulated lipolysis, an ovine primary adipocyte cell culture was required, as tissue explants are not a suitable system for antisense manipulation. However, upon investigation it was apparent that the ovine primary adipocytes developed some, but not all, of the features of the lipolytic system after the induction of differentiation. Unstimulated and isoproterenol-stimulated lipolysis developed with time after differentiation, reaching a maximum after 10 days, consistent with previous findings for GPDH activity and lipogenesis in these cells (Soret *et al.*, 1998, 1999). However, response to adenosine did not develop in this time period.

Initial studies had utilised adipocytes that were differentiated in the presence of insulin, T_3 and lipid. More recent studies have shown that the inclusion of rosiglitazone and dexamethasone, along with ex-cyte, T_3 and dexamethasone enhance differentiation of ovine pre-adipocytes (Soret *et al.*, 1999). The effect on lipolysis of

different combinations of these additions to the differentiation medium were investigated. It was immediately apparent that the cells showed little differentiation in the absence of insulin, as observed previously (Aso *et al.*, 1995; Adams *et al.*, 1996; Soret *et al.*, 1999). It was found that the maximum rate of stimulated lipolysis was observed in the presence of insulin, ex-cyte, dexamethasone and rosiglitazone, but that the addition of T₃ to this combination caused a reduction in lipolysis. This effect of T₃ was unexpected, as hyperthyroidism increases, whereas hypothyroidism decreases, response to isoproterenol in rat adipose tissue, and hypothyroidism decreased the lipolytic response to catecholamines in neonatal lambs. T₃ also causes over a 2-fold increase in β 3-adrenoceptor concentration in differentiating 3T3-F442A adipocytes due to an increase in the half-life of β 3-adrenoceptor mRNA (El Hadri *et al.*, 1996). However, although manipulation of the differentiation medium showed an increase in isoproterenol-stimulated lipolysis and sensitivity to isoproterenol under most conditions tested, no response to adenosine was observed.

As no effect of adenosine was observed in the ovine primary adipocytes, the lipolytic system in the murine pre-adipocyte cell line, 3T3-F442A, was determined with a view to using this system instead to investigate the molecular mechanisms involved in GH action. In accordance with previous findings (Dietz & Schwarz, 1991), the basal and isoproterenol-stimulated lipolytic system developed with time, reaching a maximum after 10 days of differentiation. However, as with the ovine primary adipocytes, no significant response to adenosine was observed.

No increase in the rate of lipolysis was observed after the addition of adenosine deaminase (which hydrolyses any exogenous adenosine present) in either the ovine

primary adipocytes or the 3T3-F442A adipocytes and no decrease was found on the addition of PLA, suggesting that the inhibitory adenosine A₁ receptor was either not present or not functional in these cells. It has been shown previously that, although different cell types showed a response to adenosine after differentiation, the effect was not apparent until later stages of differentiation (a minimum of 8 days) (Elks *et al.*, 1987; Ravid & Lowenstein, 1988; Saulnier-Blache *et al.*, 1991; Børghlum *et al.*, 1996). Mersmann *et al.* (1997) have also found that no inhibitory adenosine receptors are present in suckling pigs at early stages of development. It therefore seems that the inhibitory adenosine A₁ receptors are not present at early stages of adipocyte differentiation, and that their expression in the differentiating adipocytes depends on species. It would be interesting to determine the expression of the adenosine receptors at various stages of differentiation in the ovine primary adipocytes and the 3T3-F442A adipocytes. In view of the failure of the adenosine system to develop in either cell system, the investigation into the mechanism of the effects of GH on the inhibition of lipolysis by adenosine was abandoned.

Concurrent studies in the laboratory concerned the mechanism of the effects of insulin and GH on the rate of lipogenesis in differentiated 3T3-F442A adipocytes, with particular emphasis on the involvement of different PKC isoforms in the signalling pathways (Millar, 1998). The role of PKC in mediating the effects of insulin is undetermined. PKC has been associated with attenuation of the insulin response and the induction of insulin resistance (Houslay, 1991; Chin *et al.*, 1993). PKC appears to counter-regulate insulin action *via* induction of serine / threonine phosphorylation of the insulin receptor, which has been associated with decreased insulin receptor tyrosine kinase activity (see Houslay, 1991, 1994) and with decreased ability of the

insulin receptor to promote activation of the PI-3Kinase complex (Chin *et al.*, 1993). PKC activation also results in serine phosphorylation of IRS-1, which contributes to inhibition of insulin signalling (De Fea & Roth, 1997). These effects are mainly due to activation of the PKC α isoform (Chin *et al.*, 1994; Danielson *et al.*, 1995). PKC also suppresses the insulin-mediated increase in PKB activity in 3T3-L1 adipocytes (Barthel *et al.*, 1998).

However, it has been observed that insulin activates a number of PKC isoforms in adipocytes (Farese *et al.*, 1992; Bandyopadhyay *et al.*, 1997). PKC also appears to be required to mediate insulin-stimulated lipogenesis (Smal & De Meyers, 1987) and glucose transport (Standaert *et al.*, 1990; Grunberger, 1991; Yano *et al.*, 1993; Chalfant *et al.*, 1995) in the adipocyte. Therefore, mediation of the metabolic effects of insulin by PKC isoforms appears to be complex. Using antisense technology to deplete specific PKC isoforms, Millar (1998) found that in 3T3-F442A adipocytes, PKC μ is essential, and that PKC ϵ is at least partly required, for insulin-stimulation of lipogenesis. PKC μ is partly required for GH reduction of basal lipogenesis, but other PKC-independent pathways also appear to be involved. PKC γ was found to be essential for the GH antagonism of insulin stimulation of lipogenesis in 3T3-F442A adipocytes (Millar, 1998).

GH and insulin effects on the rate of lipogenesis involve alteration in the expression and activation status of key lipogenic enzymes such as ACC and PDH (Dietz & Schwarz, 1991; Bauman & Vernon, 1993; Harris *et al.*, 1993; Vernon *et al.*, 1993; Denton & Tavaré, 1995; Moule *et al.*, 1995). GH has been shown to suppress both

the activation status and expression of ACC and FAS (Dietz & Schwarz, 1991; Bauman & Vernon, 1993; Harris *et al.*, 1993; Vernon *et al.*, 1993) and prevent the activation of ACC by insulin (Vernon *et al.*, 1991). I therefore decided to attempt to determine if specific PKC isoforms were involved in the mediation of these effects on expression and activation status of ACC in 3T3-F442A adipocytes. However, despite observing an increase and decrease in the presence of insulin and GH, respectively, in the rate of lipogenesis in the 3T3-F442A adipocytes, the effects were small. There was no significant effect on either total ACC or ACC in the active state by insulin and GH, although insulin did have a slight effect on increasing the levels of ACC mRNA. A number of modifications to the original system did result in some improvement, but the size of response to either hormone was still not large enough to confidently detect any differences when looking at the different PKC isoforms.

The reason for the poor response to insulin is unclear. It was found that maintaining the differentiated 3T3-F442A cells in culture in the absence of insulin for up to 48 hours did not result in any fall in the rate of lipogenesis. This suggests that the lipogenic system is fully switched on during differentiation, and possibly is not switched off after the removal of insulin, thus leaving little scope for a further increase by insulin.

Isoproterenol was included in the studies to reduce the rate of lipogenesis, and hopefully result in a larger increase in lipogenesis in the presence of insulin. Isoproterenol did reduce the rate of basal lipogenesis in 3T3-F442A adipocytes. However, although I observed a slight increase in lipogenic rate in the presence of

insulin and isoproterenol, the effect was still small. Hence, as an alternative, the modulation of the effect on lipogenesis of isoproterenol by PDEs was explored.

In adipocytes, the intracellular concentrations of cyclic AMP are controlled by the complex coordinate regulation of both AC and cyclic AMP PDE activities. Changes in intracellular cyclic AMP greatly affect the metabolic status of the cell, with catecholamines increasing the levels of cyclic AMP, and ultimately the rate of lipolysis, while PDE's reduce the levels of cyclic AMP by hydrolysis, thereby decreasing lipolysis and increasing lipogenesis. Different PDE isoforms have been found to have different roles in cell metabolism. For example, the PDE isoform, PDE4 has been implicated in playing an important part in adipocyte differentiation (Elks & Manganiello, 1985; MacKenzie *et al.*, 1998), whereas PDE3 has been found to play an important role in regulating lipolysis in mature adipocytes (Elks & Manganiello, 1985). This suggests that the differences in response to selective inhibitors for these PDE enzymes reflects compartmentalisation of cAMP signalling (Erdogan & Houslay, 1997; Faux & Scott, 1996; Houslay & Milligan, 1997). However, while the classes of PDE involved in the regulation of lipolysis and adipogenesis have been identified, namely PDE3 and PDE4 respectively, it is still not clear which PDE enzymes have a role in modulating the rate of lipogenesis.

Using selective and non-selective inhibitors in the presence and absence of isoproterenol, I found that both PDE3 and PDE4 appeared to be involved in modulating lipogenesis. Other findings have indicated that more than one PDE enzyme is involved in the regulation of lipolysis, although PDE3 has the most effect (Arner *et al.*, 1993). I found that none of the selective PDE enzyme inhibitors used,

either together or individually, had as much of an effect on lipogenesis as IBMX (a non-selective PDE inhibitor) had. However, in the presence of isoproterenol, the combination of PDE3 and PDE4 inhibitors achieved a similar effect on lipogenesis as the non-specific inhibitor, IBMX. Thus, the pool of cyclic AMP involved in the inhibition of lipogenesis may be different to the one involved in the stimulation of lipolysis by catecholamines.

The failure of the antilipolytic system of adipocytes to develop during adipogenesis and the poor response of the differentiated 3T3-F442A adipocytes to insulin and also GH truncated my ambitions to investigate the intracellular signalling system, in particular the role for specific isoforms of PKC, in the chronic metabolic effects of GH and also insulin. The study has, however, shown that effects of GH on adipocytes are not mediated by $\text{TNF}\alpha$. Furthermore, effects of a number of factors on the development of the lipolytic system of ovine adipocytes have been identified, and a role for both PDE3 and PDE4 enzymes in the modulation of lipogenesis has been demonstrated. For the future, it may be possible to use differentiated ovine pre-adipocytes to further explore the roles of PKC isoforms in the control of lipogenesis by GH and insulin, as these cells have a much greater response (10-fold in the case of insulin) to the hormones than 3T3-F442A adipocytes. However, whether the ovine cells are susceptible to antisense manipulation is not known, and the appropriate oligonucleotide sequences for such studies would need to be defined. Whether a suitable cell culture system for investigating the effects of GH in the response to adenosine can be established remains uncertain as the reason for the lack of response to adenosine requires to be determined first.

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